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Review Article

Advances in Nanocomposite-Based Fertilizers for Sustainable Agricultural Practices

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Abstract: Nanotechnology is having a significant impact in the field of agriculture, providing solutions to increase agricultural output to solve environmental issues. It promotes sustainable agriculture and successfully solves the current crisis of global food security. Sustainable agriculture is vital for securing food deliveries, maintaining resources, and combating environmental impact. The objective of this review is the recent development of nanocomposites based fertilizers in promoting agricultural sustainability. The use of traditional agricultural fertilizers indicates high nutrient losses of 80-90% of phosphorus and 50-70% of nitrogen contributing to environmental pollution. To cope with these demands, advancement in nanotechnology, such as nanocomposites-based fertilizers, is a substitute. Prominent trials in field scale indicate that zinc oxide nanocomposites enhanced crop yields by 25-30% though reducing fertilizer application rates up to 50%. Chitosan based nanocomposites demonstrated dual benefits of improved nutrient uptake and disease resistance, increasing plant biomass by 15-20%. The Nanocomposites based fertilizers are ecologically friendly, reduce environmental issues, provide sustainability to agriculture and agronomy, promote crop productivity, reduce the wastage of traditional fertilizers, and improve soil health. This review is estimated to cheapen and transform agricultural practices, increasing sustainability and productivity for shareholders and all associates for upcoming generations.

Keywords: Nanocomposites Fertilizers, Sustainable Agriculture, Controlled Nutrient Release, Eco-friendly Farming Solutions.

1. INTRODUCTION

The term "nanotechnology" originates from the Greek word "nano", which means to "extremely small". It covers a billion meters [1]. So, we know nanotechnology is a term used to understand and organize the substances ranging from 1-1000 nm which is an emerging technology of the 21st century [2]. Nanotechnology is a technology that we implement at the nanoscale and has a lot of applications worldwide. Nowadays nanotechnology has turned out to have a general purpose representing a megatrend that benefits the public [3]. Currently, in agriculture nanotechnology applications improve soil health, plant mineral nutrition (uptake of minerals), reduce fertilizer waste, enhance crop products, and micro-flora with soil, leading to sustainable solutions to replace bulk fertilizers [4]. Nanotechnology based management of crops is a very vital tool for improving agricultural yields. Quantum dots, nanofibers and carbon nanotubes of nanostructure and nanomaterials are used in agricultural research as biosensors to check the distribution of fertilizers and quality of the soil. In improving quality of crops, the carbon nanotubes and nanomaterials of TiO_2, ZnO, SiO_2, and gold are used [5, 6]. Additionally, nanotechnology specifically biogenic silver nanoparticles, holds

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immense promise across various fields, their distinct physiochemical attributes for potential applications as potent antimicrobial and anticancer agents [7, 8]. Agriculture is a significant growth drive all over the area, contributing to local increase of production [9]. For the survival of the rapidly growing global population, plant species treated with nano fertilizer must exhibit tolerance towards climate change [10]. In the growth of the economy, agriculture may contribute in major proportions which means that somehow 40-60% of the countrywide income is produced by agriculture and 50-60% of people are doing job in the production of agriculture [11]. In agriculture, nanotechnology is forwarding step in the direction of tolerance and agricultural products through solubility enhancement, control release and targeted delivery while also lowering the damage [12]. Agricultural crops are prime food sources and vital fields of our living organisms which feed our increasing populations. Plant diseases have currently become a major factor in the deterioration of food quality and production, so to overcome this problem, nanotechnology is a promising area in agriculture from seed sowing in soil the cultivation of plants [13]. Nanotechnology presents promising avenues for sustainable agricultural practices, wherein nanochemicals play a pivotal role in enhancing plant growth and managing pests. Nanomaterials, like silver nanoparticles, are also used in food packaging to keep microbes from getting into the food. However, they need to be carefully studied to see how they might affect plant growth before they can be used legally [14, 15]. Amidst the challenges posed by climate change, advanced nano-engineering emerges as a crucial ally in enhancing crop production and securing agricultural sustainability.

Nanotechnology contributes to agricultural efficiency by optimizing input utilization and minimizing losses, particularly by leveraging nanomaterials to enhance fertilizer and pesticide delivery. Moreover, nano biosensors enable precise management and control of agricultural inputs, facilitating the adoption of high-tech farming methods [16]. Nanotechnology, which uses nanomaterials as carriers, facilitates a variety of applications in plant growth and crop management, including nanofertilizers, nanopesticides, nanosensors, and nanobiotechnology. Nanotechnology enhances the effectiveness and durability of agro-chemicals by harnessing distinctive structural properties, fostering plant growth and resilience to environmental stressors [17].

In the burgeoning era of nanotechnology's integration into agriculture, a rich tapestry emerges, illuminating the intricate interplay between nanomaterials and the soil-plant interface. From the artistry of synthesis to the orchestration of metabolic pathways, nano fertilizers hold promises for enhancing crop physiology while potentially reducing dependency on pesticides through heightened reactivity. As attention turns to nanoparticles used in pesticides and soil restoration, new areas are opening that haven't been explored yet. This is made even more important by the need to quickly figure out how soil nanomaterials move and what effects they have on farming systems [18]. It increases the ability of plants to resist abiotic and biotic stress by improving yield and quality of crops through the process of gene editing, and nanotechnology also contributes to the connotation of CRISPR/Cas [19]. Elimination of pollutants has become a major problem worldwide so to combat this issue nanotechnology is used to boost up the process of bioremediation. Nano remediation is a promising solution to this manmade disaster [20]. Nanotechnology can also modify the genome of the mitochondria and plastid as well as edit germ lines [21].

Agricultural lands are facing various critical challenges that threaten their productivity and sustainability. The primary and major problem is low resource use efficiency, specifically for fertilizers and water. The use of conventional fertilizers often displays poor nutrient use efficiency due to runoff, leaching, and volatilization with more than 50% of applied nutrients being lost [22]. This contributes to environmental pollution, i.e. increased greenhouse gas emissions; eutrophication of water bodies and results in significant economic losses. Moreover, overuse of chemical fertilizer has led to degradation of soil, lowering organic matter in soil, disrupt diversity of microbes, and deteriorating soil fertility for long term. Scarcity of water is another pressing problem, as agriculture consumes the largest share of global water, and ineffective irrigation practices. Likewise, over dependency on chemical pesticides has faster pest development and disease resistance, requiring the rising production costs and higher inputs [23]. These challenges are compounded

by the impacts of climate change, comprising of droughts, floods, and unpredictable weather patterns that decrease crop productivity and increase the risk of food insecurity. The rapidly growing population of the world is expected to reach 10 billion by 2050, guaranteeing sufficient production of food and protecting environmental health has become an urgent priority [24]. This study aims to investigate advancements in nanocomposite-based fertilizers recently, increased nutrient use efficacy, directing on nutrient release mechanism, and possibility of environmental benefits. In addition, it also seeks to promote awareness among researchers, farmers, and experts to emphasize the need for commercialization and Environmental Impact Assessment for sustainable agricultural practices.

2. METHODOLOGY FOR SEARCHING

2.1. Information Retrieval Approach

We conducted a detailed search on electronic databases such as Google Scholar, Science Direct, PubMed, and Scopus to find the most relevant literature related to our review topic. We used specific phrases and keywords like "nanotechnology", "nanofertilizers", "nanomaterials", "traditional fertilizers", "smart fertilizers", "nanocomposite materials-based fertilizers", and "agricultural sustainability" during present search. This approach ensured that we gathered comprehensive data from 2000 to 2024 information for this study.

2.2. Inclusion Criteria

- Articles that target nanocomposite fertilizers and their applications in agricultural sustainability.
- Examines environmental benefits of nanocomposites and controlled nutrient release mechanisms.
- Research exploring synthesis sources and bio nanocomposites in agricultural applications.

2.3. Exclusion Criteria

Research articles not related to nanocomposites applications in agricultural and only on traditional fertilizers without nanocomposite.

2.4. Detailed Analysis Taken Articles

One of the authors conducted an individual revision to the full articles acquired through electronic search. Relevant data from these articles were extracted, and any discrepancies were resolved through discussion and then referred to the second reviewer (another author) for a final decision. The extracted data were then summarized and organized in (Table 1) showing diverse nanocomposites material synthesized from plants, used primarily for antimicrobial activity, catalysis, plant growth enhancement, and environmental remediation and (Table 2) showing nanocomposites material synthesized from various plants source for antimicrobial, growth enhancement, and photocatalytic applications in agriculture for comprehensive analysis. The details from the extracted data were explained under corresponding headings and shown in Figure 1 focus on the role of nanocomposite materials in sustainable agriculture by improving fertilizers, soil management, pesticides, stress management and food security and classifies nanofertilizers on the basis nutrient content, release mechanisms, and special coatings for controlled nutrient delivery to provide a clear demonstration (Figure 2).

3. RESULTS AND DISCUSSION

3.1. Nanocomposites Material

Nanocomposites are materials that are composed of nanosized standard substances. It is characterized by chemical composition, physical characteristics, resource, external appearance, and size [25]. Bio nanocomposites are minute in size, ranging from 1 to 100 nm in the large amount of similar elements, but have the same nano characteristics. Bio-nanocomposites are minute in size, ranging from 1 to 100 nm in the large amount of similar elements, but have the same nano characteristics. Bio-nanocomposites in agriculture have a lot of applications, including plant growth, crop production, pest protection, and providing good nanoparticles or agricultural chemicals [26]. Nanocomposites are solid substances comprising multiple phases, with each phase exhibiting dimensions that may span one, two, or three proportions in the size of a nanometer [27]. Agriculture is facing challenges in terms of sustainability and population growth which prompt innovation such as polymeric films using the non-biodegradable polyethylene. Bio-nanocomposites play a key role by improving the mechanical properties and allowing the controlled release of

Table 1. Nanocomposites-based material synthesized from plant sources and their multifaceted roles across various applications.

S. No.	Nanocomposites material	Source of Synthesis	Size/wt	Roles	References
1	ZnO-rGO	*Avicennia marina* and *Polycladia myrica*	28.1 nm	Fouling resistant activity.	[31]
2	CdS/CQDs	*Aegle marmelos*	73 nm	Photocatalytic activity.	[32]
3	Fe_3O_4/HAP/ZnO	*Falcaria Vulgaris Bernh*	500 nm	High porosity level. Separation of substances from aqueous solution.	[33]
4	Ag/Bhm NC and Ag/Bhm/Chit NC	*Rosmarinus officinalis*	72.3 and 60.8 nm	Apoptotic and sporicidal agents.	[34]
5	ZnO-Chitosan	*Azadirachta indica*	39 nm	Antibacterial activity and UV protection.	[35]
6	Mn-ZnO	*Withania somniferum*	11-14 nm	Reduce environmental pollution.	[36, 37]
7	Ag-doped ZnO/MgO	*Caccinia macranthera*	88 nm	Use for detection lead ions.	[38]
8	Ag and ZnO, Silver-peptide	*Trigonella foenumgraecum*	75 nm	Antimicrobial activity against *Staphylococcus aureus* and *E. coli*. Antifungal activity against *Candida albicans.* Also act as photo catalyst and antioxidant agent.	[39, 40]
9	ZnO and TiO_2	*Hibiscus*	10/90 and 50/50 wt	Used as protecting agent in lime mortars and stones coating.	[41, 42]
10	Ag and ZnO	*Silybum marianum*		Biomedical	[43]
11	$BiVO_4$	*Hyphaene thebaica*	75 nm	Biomedical	[44]
12	BCN	*Saccharum officinarum*	2-100 nm	Increase mechanical vigor and pure type of NC.	[45]
13	Au and Ag alloy	*Azadirachta indica*	50-100 nm	Help in remediation of heavy metals.	[46]
14	CNF	*Elaeis guineensis* *Beta vulgaris* L. and *Gossypium* species	3-60 nm	Used in packaging, foams, paints films and coating.	[47]
15	Bimetallic Ag and Au	*Punica granatum*	12 nm	Reduction of nitrogen compound into aromatic amines. Act as antioxidant agent.	[48]
16	ZnO and CeO_2	*Acacia nilotica*	12 nm	*S. aureus* and *K. aerogenes* shown antibacterial activity. Help in degradation of methylene blue dyes.	[49]
17	MgO and Cu	*Cassytha filiformis* L.	12 nm	Reduce methylene blue, nitrogen compounds and Congo red by catalytic activity.	[50]

18	CuO and C	*Adhatoda vasica Neea*	7-11nm	*Candida albicans and Aspergilus niger* showing antifungal activity. *S. aureus, K. pneumoniae, E. coli*, and *P. aeruginosa* showing antimicrobial activity.	[51]
19	SiO-TiO_2	*E. coli*	0.8 × 5 mg	Role in microbial activity, osteogenesis, and LED (Light emitting diode).	[52]
20	ZnO and FeO BNC	*Anethum graveolens*	15/15 wt	Enhance seed biomass, vegetative growth and inflorescence and improve stress tolerance in plants.	[53]
21	Ag-ZnO	*Tetradenia riperia*	200-600 µg/m	Antibacterial activity	[54]
22	Alginate/Chitosan with CuO	*Fortunella margarita*	300 nm	Enhances the germination of seed.	[55]
23	Vinasse Biochar dolomite	*Sectaria viridis*	41 nm	Phosphorus fertilizer substitute and enhance growth.	[56]
24	Bentonite/ Acrylate acid-co-acryl amide	*Oryza sativa*	34.6 nm	Use in agriculture for water absorption and nitrogenous (urea) fertilizers.	[57]
25	CNCs (Alginate coated cellulose nanocrystals)	*Gossypium arboreum*	5% wt	Increase Hydrogel property. Improve water retention Help in sustainability of fertilizers.	[58]

agricultural chemicals, promoting cultivation, and decreasing pollution by facing obstacles in commercialization such as production costs and nontoxicity [28]. Nanocomposites lessen the pesticide and fertilizer burden, offering promising solutions for sustainable agricultural practices [29]. Nanocomposites have significant properties that are related to native polymers formed by the addition of nanoparticles to polymers. Primarily, it controls the spatial distribution of nanoparticles. Secondly, predicting the spreading of particles and the condition of organization can optimize many properties of nanocomposite and lastly, we must look at the function that particle nature (form) affects dispersion therefore controlling the property. Nanocomposite has achieved good attention in advancement of agriculture products that are based on nanotechnology [30].

3.2. Agricultural Fertilizers and their Importance

Agricultural fertilizers are compounds that are composed of a mixture of elements and essential mineral substances used for plant nourishment and growth [59]. Currently, to make agriculture sustainable and productive, fertilizers are among the most significant [60]. To cope with the demand for food due to the increase in population, fertilizers are essential agricultural inputs. Complications of human health problems and many environmental issues have resulted from the application of careless use [61].

3.2.1. Smart fertilizers

Smart fertilizers are used in top, which is applied for the management of interval, time, and speed of nutrient release and absorption by plants for improving crop growth and reducing the impact of other environmental issues [62]. In smart fertilizers, one is slow-release fertilizers (SRF), which refers to the spreading of nutrients that are available to plant life through the processes of solubility reduction, biodegradation, and hydrolysis as compared to products that are reference soluble,

Table 2. Applications of Nanocomposites material in agriculture.

S. No.	Nanocomposites material	Source	Size	Roles	References
1	Ag-chitosan	*Solanum lycopersicum*	87 nm	Control bacterial wilt	[73]
2	Ag-Starch	*Punica granatum*	1-54 nm	Antimicrobial activity	[74]
3	Carboxymethyl Cellulose/Silver (CMC-AgNPs)	*Syzygium aromaticum*	30-70 nm	Antibacterial activity	[75]
4	Mt-Ag	*Zea mays*	10.52 nm	Wastewater treatment	[76]
5	Ag-chitosan	*Cissus arnottiana*	23 nm	Antibacterial activity	[77]
6	AgNP/MCC/starch/whey protein	*Azadirachta indica*	20 nm	Antibacterial and food preservation activity	[78]
7	Ag-Chitosan	*Aloe vera*	20 nm	Improve physiochemical properties	[79]
8	Zinc oxide/carbon nanofiber	*Thymus daenensis and Stachys pilifera Benth*	45 nm	Efficient Antibacterial and photocatalytic activity	[80]
9	Zinc oxide–silver Zinc oxide–copper	*Bridelia ferruginea*	18.98 and 18.90 nm	Improve phenolic and terpenoids	[81]
10	Zno-GO	*Sonchus Oleraceus*	5 nm	Generation of Reactive Oxygen Species	[82]
11	ZnO-Ag	*Thymus vulgaris*	75 nm	Antioxidant, photocatalytic and antimicrobial activity	[83]
12	$Mg0.5Zn0.5FeMnO_4$	*Astragalus gummifer*	20 nm	Photocatalytic activity	[84]
13	ZnO-NiO	*Sterculia foetida*	30-35 nm	Antibacterial activity	[85]
14	Chitosan-ZnO	*Beta vulgaris*	20-80 nm	Antibacterial activity	[86]
15	Ag-ZnO	*Ocimum tenuiflorum*	30-40 nm	Dye degradation and antibacterial	[87]
16	Ag-ZnO	*Trigonella foenum-graecum*	75 nm	Antioxidant and antifungal	[88]
17	TiO_2-PN	*Ageratina altissima*	60-100 nm	Increase catalytic activity	[89]
18	Ag-TiO_2	*Cymbopogon citratus*	41.8 nm	Catalytic and antimicrobial activity	[90]
19	TiO2–ZnO	*Allium sativum*	4.58 nm	Improve growth parameters	[91]
20	Ag-TiO_2	*Vitis vinifera*	10 - 30 nm	Bactericidal, Antioxidant, Photocatalytic, and Activities	[92]
21	Ag-TiO_2	*Cymbopogon citratus*	49 nm	Metal Degradation	[93]
22	Cu-Chitosan	*Zea mays*	37 nm	Increase seedling growth	[94]
23	Chitosan-Zn Chitosan-Cu	*Oryza sativa*	56 nm	Protective agent and improve plant growth	[95]
24	Cur-Chitosan	*Pyricularia grisea*	34.6 nm	Biocontrol agent against blast disease and increase plant growth	[96]
25	K-Chitosan	*Zea mays*	39 - 79 nm	Improve overall growth and act as soil conditioner	[97]
26	Se-Chitosan	*Raphanus sativus*	50 nm	Enhance uptake of essential nutrients	[98]
27	Cu-Chitosan	*Zea mays*	36 nm	Act as fungicide	[99]
28	Chitosan-Ag	*Capsicum annuum*	4 nm	Antifungal agent against *Colletotrichum truncatum* causing anthracnose	[100]
29	Chitosan-Metal	*Prunus avium*	76 nm	Agro fungicides	[101]
30	Graphene-tinO	*Cocos nucifera*	3 nm	Antibacterial activity	[102]

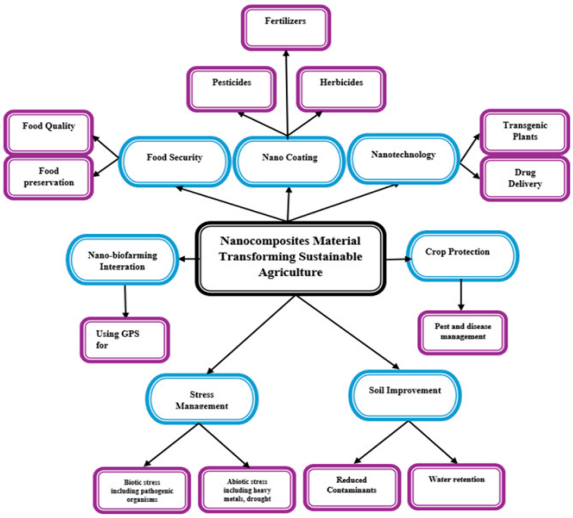

Fig. 1. Flow chart of Nanocomposites-Based-Material Transforming Sustainable Agriculture. This flowchart is obtained by using Bio render software.

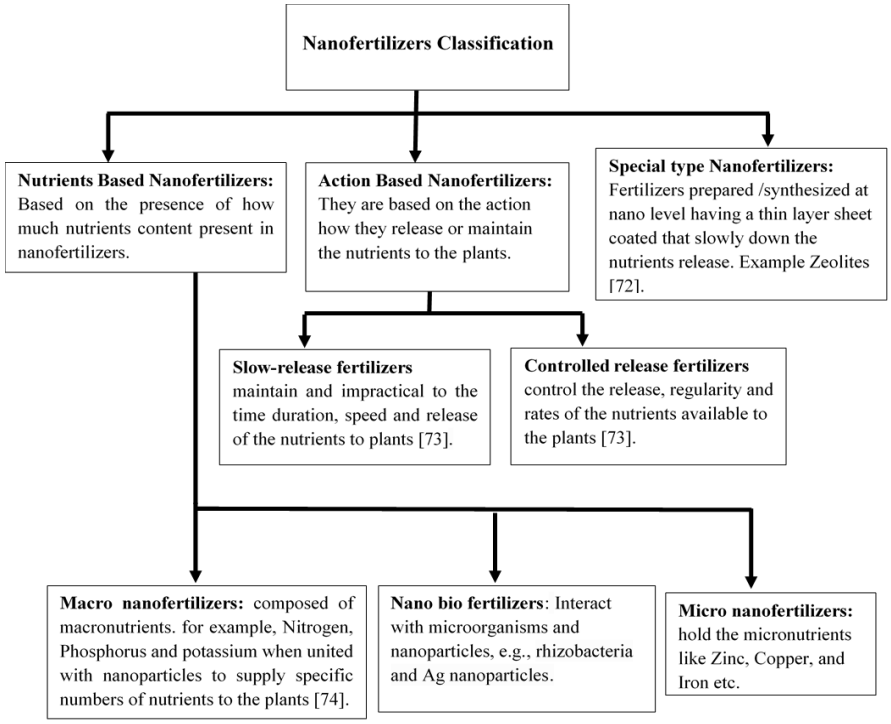

Fig. 2. Classification of the nanofertilizers on different basis like nutrients based, action based, and special type are categorized above.

like urea, ammonium nitrate, and ammonium sulfate. These fertilizers are commonly used for nitrogen containing compounds that participate in the breakdown of bacteria, e.g., manure which is available to us naturally, but we cannot synthesize in controlled conditions. SRF can be in two forms of either organic or inorganic, which release nutrients slowly over time. Conclusively, SRF are traditionally used fertilizers that progressively release nutrients for a long period of time and are not that environmentally destructive [63]. The other one is controlled release fertilizers (CRF) that refers to fertilizers which release nutrients when met with necessary factors like rate and period of nutrients and temperature. CRF holds nutrients that are not readily absorbed by the plants. We use organic or inorganic compound coatings on CRF to regulate the duration, pattern, and rate of nutrient liberation for the plants [64].

3.2.2. Nano fertilizers and their classification

Nano fertilizers have led to research for eco-friendly fertilizers, specifically with having high nutrient use efficiency whose alternative promise is emerging nanotechnology. Application of these nutrients either separately or collectively bound to nano based adsorbents, which will release the nutrients deliberately compared to other fertilizers. Through this approach, we can reduce the leaching of nutrients in ground water and enhance nutrient use efficiency [65]. The overuse of chemical fertilizers and pesticides nowadays has led to severe human health problems and environmental pollution. Nowadays, nanoscience might solve these problems by providing nanofertilizers, i.e., nitrogen, phosphorus, potassium, zinc etc., [66-70].

3.3. Mechanism of Nanocomposites Root Delivery Improving Nutrient Uptake Efficiency

Nanocomposites material is made to measure nutrients that are delivered precisely to plant roots. Their tiny size and roomy surface area allow them to penetrate inside the roots effectively, ensuring direct access to the root zone where nutrients are highly required. This targeted delivery system ensures plants receive nutrients exactly where and when they need them, promoting optimal growth. In terms of nutrients efficiency, nanocomposites play a key role in improving how plants absorb nutrients. The special characteristics of these materials is that they increase the solubility and availability of nutrients, making them easier for plant roots to access. Moreover, the large surface area of nanoparticles facilitates better interaction with root surfaces, leading to enhanced nutrient absorption. This improved efficiency means plants can make the most of available nutrients, resulting in healthier growth and development [71].

The exact mechanism for slow-release fertilizers works in two steps: Firstly, through photo degradation, where exposure to sunlight, soil temperature changes, and cultivation practices cause the fertilizer to crash, releasing nutrients. Secondly, in biodegradation, soil microbes break down the fertilizer, converting it into forms that plants can readily absorb, ensuring nutrient availability for plant growth. While controlled release fertilizers utilize various mechanisms to release nutrients effectively where nutrients gradually permeate the coating material, maintaining a stable release when the water potential stabilizes. This process guarantees a consistent supply of nutrients, with coating permeability and temperature playing pivotal roles. Secondly, osmotic pumps and fertilizers couple osmotic pressure alterations, influenced by variations in hydrostatic pressure and osmotic gradients for nutrient release. Pressure accumulation causes the coating to become semi permeable and develop cracks, releasing solutes and delivering nutrients to the plant as needed. Lastly, convective releasing under higher hydrostatic pressure, the coating can rupture, resulting in a swift release of nutrients. This phenomenon is commonly seen in fertilizers such as sulfur coated urea, primarily stems from coating breakdown, guaranteeing prompt nutrient availability for plants [72].

3.4. Nanocomposite-Base Fertilizers as the Future Frontier of Sustainable Agriculture

The efficiency of nutrient based fertilizers is low because of the various pathways of losses: runoff, fixation, immobilization of microbes, denitrification, and leaching. Estimations of these nutrients are that more than 95% of micronutrients, 80-90% of phosphorus, 50-70% of potassium, and 40-70% of nitrogen are lost in our environment, which alternatively results in pollution. Smart fertilizers like bio formulated fertilizers such as nanofertilizers and slow-release fertilizers are advanced technologies that increase the efficiency of using nutrients and improve crop production in a sustainable way. To make use of slow, controlled release fertilizers, it reduces eutrophication, contaminants in water, leaf burn risk and the efficiency of nutrient use [103]. Nanofertilizers are vital in reducing the use of agrochemical fertilizers and minimizing their aggressive effects on the environment. Nanofertilizers can penetrate the epidermal layer of plants because of their reactive nature, which increases the efficiency of nutrients and decreases the excess nutrients, leading to productive crops [104]. Chitosan is an amino polysaccharide that is regarded as the next generation of fertilizers that enhance the immune systems of plants by delivering nutrients in a controlled, slow, and targeted way. It can help researchers in the agricultural system and is applied in multiple fields as an effective and ideal preference [104]. Nanofertilizers also improve

the health of soil by releasing bounce nutrients, improving the improving the structure of soil, and increasing the activity of valuable microbes [105]. Silicon, which is a metalloid element, affects the growth of plants, manages stress, and improves crops. The application of Si (available to plants in Orthosillicic acid) helps with soil fertility, moisture content and nutrient uptake, resulting in improved growth, yields and defense against stress [106].

For increasing food production mineral fertilizers are key despite heavy losses and low uptake of nutrients, yet nanofertilizers can lead to increased crop production and a reduction of nutrient loss. This increases the attention given to nano based fertilizers, which is the concept of nanotechnology. So marketable nanofertilizers can lead to the sustainability of agricultural trade [107]. The limits of conservative products which are biodiversity loss, human diseases and environmental issues are overcome by the applications of controlled release systems (nanocarriers). Nano carriers are ecofriendly and sustainable strategies for agriculture, ecosystem and human fitness [108]. Synthetic NFs are an essential input and demanding for current agriculture [109]. Nanofertilizers revealed the ability to add to the production of food materials, civilizing nutritional food and tumbling waste substances to strengthen the sustainability of agriculture products [110, 111]. Nanofertilizers are intended for specific targets and do not cause disturbances in the environment. It can help to enhance crop production, increase the use of nutrients and lessen the use of unwarranted synthetically prepared fertilizers [112]. Agronomical applications of nanocomposite material are used as nano-sensor, nano-pesticides and nanofertilizers which facilitate the release of pesticides and smart fertilizers to lessen the discharge of toxic substances into our surroundings [113].

3.5. Navigating the Challenges of Traditional Fertilizer Usage

The usage of traditional fertilizers poses a challenge to eco efficient agriculture because of its involvement in environmental degradation, resource inefficiencies, and economic burdens. To address these challenges, it requires a transference towards sustainable practices that optimize nutrient management, embracing precision agriculture, and incorporating organic fertilizers to increase soil health and decrease the environmental impact. Holistic approaches encompassing social, economic, and institutional dimensions are essential for fostering eco efficient agricultural systems, and crucial for long term food security amidst global demands and environmental pressures [114]. Also, traditional fertilizer usage presents a dual challenge: significant ammonia emissions contribute to environmental pollution and health risks. To tackle this, we must boost fertilizer efficiency, curb environmental impact, and navigate the delicate balance between food production and biofuel needs on available arable land. Engaging policymakers and stakeholders in a thorough discussion is vital to setting targets that foster sustainable agricultural practices, ensuring food security while safeguarding the environment [115].

Dealing with the problems of conventional fertilizer application for boosting agricultural output, the traditional fertilizers use substantially contributes to nitrous oxide emissions and global warming [116]. The widespread utilization of conventional nitrogen fertilizers poses significant hurdles, as around 50% of the nitrogen input escapes into the environment, leading to air and water contamination and posing risks to food and environmental safety. Forecasts suggest a 150% rise in nitrogen pollution by 2050, with agriculture exerting a notable influence. [117]. Both inorganic and organic fertilizers play critical roles in nourishing crops and improving soil quality, but they also carry substantial pollution risks from potential contaminants and mishandling. Nitrogenous and phosphate fertilizers are major contributors to soil, water, and air pollution, which in turn affects human health and worsens climate change by releasing greenhouse gases. Moreover, fertilizer misuse leads to water bodies becoming eutrophic and heavy metals accumulating in the soil [118].

3.6. Transforming Agriculture with Smart Fertilizers

Agricultural challenges feeding a highly growing population and fighting against climate change, stress the importance of efficient, affordable, and environmentally friendly fertilizers. It delves into the concept of smart fertilizers and nanocomposites material, which control nutrient release, particularly by examining field scale studies on herbaceous

plants. Smart fertilizers are formulations that adjust nutrient release timing to meet plant needs, ultimately enhancing yields and sustainability in contrast to traditional fertilizers [119]. As climate change poses a growing threat, there is a pressing call for inventive ways to tackle its impact on agriculture. Smart fertilizers emerge as a key solution, helping to counteract nutrient shortages worsened by shifting environmental patterns. Using precision agriculture methods, these fertilizers finely tune distribution using precision agriculture methods, boosting crop strength, and reducing environmental harm. This blend of technology with sustainable farming methods holds great promise for addressing the agricultural challenges brought on by climate change [120].

3.7. Nanocomposite for Improved Nutrient Delivery and Crop Yield

Nanotechnology modernizations deliver sustainable and robust agricultural solutions, promoting both crop yield and quality while protecting food security. Despite progress in nano fertilizers, pesticides, and delivery systems, ongoing study is vital to creating biodegradable, affordable, and safe nanomaterials, underscoring the need for systematic studies and public awareness campaigns to protect food production systems [121]. Adding nanocomposite materials to smart fertilizers addresses discourses the challenges impersonated by excessive usage of traditional fertilizer, which promotes soil health and optimizes nutrient absorption in plants. Engineered nanocomposites facilitate precise, controlled release delivery of agrochemicals, catering to specific crop requirements. These strategies will sustain and target the release, emphasizing their significance in boosting plant growth and defense which would prioritize scalability, cost efficiency, field testing, and environmental safety as imperatives for the wider adoption of nanocomposite-based smart fertilizers [122]. This integration of nanocomposites material offers a hopeful path toward sustainable agriculture, undertaking ecological issues and stimulating food security. These cutting-edge nano formulations elevate nutrient delivery, soil vitality, and crop productivity, all while curbing environmental harm [123].

3.8. Nanocomposites in Disease Management

Nanocomposites revolutionize agriculture by combating crop diseases through smart fertilizers. These fertilizers utilize nanotechnology for precise nutrient delivery and real time disease detection, ensuring targeted efficacy. By continuously monitoring plant health and adjusting nutrient delivery, they offer a proactive approach to disease management, promising sustainable agricultural productivity [124]. Bio-nanocomposites (BNCs) and endophytes showcase unique nanoproperties and have various applications, notably in agriculture, where they bolster plant defense against pests, growth, and crop yield [125, 126]. Nanocomposites are key to fighting against plant diseases with cutting edge methods. By merging nanotechnology with biopolymers, these hybrids boast improved mechanical and thermal attributes, proving invaluable in disease control particularly silver nanoparticles, graphene oxide, and chitosan-based blends that display strong antifungal properties against pathogens like *Rhizoctonia solani, Fusarium graminearum,* and *Botrytis cinerea,* presenting hopeful avenues for agricultural disease management [127]. It also controls the disease by providing dual benefits including nanopesticides that protect plants from phytopathogens, while nano fertilizers enhance plant growth, which is crucial for global food production [128]. Nanocomposite-based smart fertilizers transform crop disease management by delivering nutrients precisely and detecting threats promptly. Through the incorporation of nanoparticles with high surface to volume ratios, these fertilizers ensure targeted delivery of disease fighting agents. Integrated nanosensors enable real time disease detection, facilitating swift intervention. Moreover, the engineered release of bioactive compounds triggers plant defense mechanisms, providing comprehensive protection against crop diseases [129].

Chitosan, known for its versatility, is widely utilized in pharmaceuticals, biomedicine, and agriculture due to its cost effectiveness and advantageous properties. Acting as a carrier for diverse active substances, including nanomaterials and essential oils, aids in improving plant nutrient absorption and bolstering defense mechanisms against pathogens. Through various production techniques, chitosan-based nanocomposites offer solutions like controlled release of nutrients and pesticides, along with antimicrobial effects against bacteria, fungi, and viruses [130, 131]. These

fertilizers ensure optimal nutrient and disease-fighting agent delivery to plant roots, maximizing absorption. Antimicrobial agents embedded within the nanocomposites are released gradually, curbing pathogen growth and lessening disease impact [132].

3.9. Future Perspective of Nanocomposites-Based Fertilizers

Fertilizers made from nanocomposites offer hopeful remedies for the sustainability issues in agriculture. However, their broad acceptance faces obstacles due to worries about scaling up production and ensuring their durability over the long term. Carbon based and metal/metal oxide nanoparticles show promise in enhancing membrane functionality, particularly in preventing fouling and enhancing permeability. Future research ought to prioritize optimizing nanoparticle concentration to prevent aggregation, tackling scalability hurdles for real world application, and thoroughly examining the long-term durability of these membranes [133]. Intensive research on two dimensional nanomaterials like MXenes, metal dichalcogenides and graphene is driven by their exceptional properties, which offer promise in agriculture and food solutions. Potential applications include sustainable water management, advanced agrochemicals, nanosensors, and packaging, with significant benefits such as enhanced purification and sensitive contamination detection [134]. The potential of nanostructured graphene materials to enhance PV efficiency is due to their high electron mobility and conductivity. Incorporating semiconductor metal oxides with graphene further enhances efficiency by facilitating photon absorption and reducing recombination centers. The synthesis of high-quality graphene and graphene/metal oxide materials is crucial for advancing PV technology and stimulating innovation in sustainable energy solutions [135]. Chitosan based nanocomposites, which combine chitosan (CS) with metal oxide nanoparticles, show great promise in identifying hazardous metal ions (HMIs) in water. These materials trigger detectable changes in either the light they reflect or their ability to conduct electricity due to chemical interactions called chelation. CS brings advantages like being friendly to living tissues and easy to squeeze, making these nanocomposites potentially affordable for spotting HMIs effectively. Despite these benefits, challenges like needing to be very sensitive, staying stable, avoiding interference from other substances, and consistently giving the same results need to be addressed. Take, for example, an innovative electrochemical sensor that utilizes a blend of CS, reduced graphene oxide (rGO), and titanium dioxide (TiO_2) to spot lead (Pb (II)) with precision. This sensor has an impressively low limit for detecting lead and works well in various situations, from checking environmental and biological samples to even testing food, showcasing its adaptability [136].

4. CONCLUSIONS

Nanofertilizers are used the aim of improving agricultural sustainability and increasing crop yields. Nanocomposite-Based Fertilizers propose substantial potential for increasing agricultural sustainability by improving crop yields and nutrient use efficiency whereas reducing fertilizer application. This approach also encourages the use of environmentally friendly methods in modern agriculture. The combined use of nanotechnology and nanocomposites with agronomy, particularly in the application of nano smart fertilizers, promises an attractive pathway to improving agricultural productivity and mitigating environmental problems. Nanofertilizers are available in various forms, including nano biofertilizers, micro nanofertilizers, and macro nanofertilizers. Each type provides nutrients to support plant growth. Furthermore, smart fertilizers such as controlled release fertilizers (CRF) and slow released fertilizers (SRF) contribute to effectively regulating nutrient levels and minimizing environmental impacts compared to conventional fertilizers. In addition, nanofertilizers are designed to target specific areas, resulting in limited damage to the environment and the most beneficial production of crops. They are involved in the development of agronomic technology, which contributes to solving food security issues, promotes ecological sustainability, and resolves the challenges faced by modern agriculture.

5. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Biosynthesis of Copper Nanoparticles by Cultures from Collection of Microorganisms

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Abstract: Biogenic production of copper nanoparticles by bacteria and fungi presents certain scientific and applied interest. In this regard, the ability to synthesize copper nanoparticles by microorganisms from the culture collection of the Institute of Microbiology, which were isolated from polluted areas, was studied. Twenty-one fungal strain and eight bacterial strains were screened, and the biosynthetic activity of representatives of *Pseudomonas* genus, as the most active biosynthetic of copper nanoparticles, was studied in a comparative aspect. The biosynthetic activity was determined 24-72 hours after adding standard solutions of $CuSO_4$ of different concentration (25-50 mg/l by Cu^{++}) to the culture liquid. The formation of nanoparticles was recorded by changing the color of the solution, as well as using UV spectroscopy and atomic force microscopy (AFM). Two strains of *Fusarium oxysporum* and one strain of *Penicillium* sp. were found to be the most active among the tested micromycetes. The formation of cubic copper nanoparticles and needle-like structures with a diameter of 800 nm and a length of 40 microns, which were formed as a result of aggregation of cubic nanoparticles with a size of 300-400 nm, has been established. All the tested bacteria showed the ability to synthesize copper nanoparticles, while *Pseudomonas stutzeri* and *Pseudomonas* sp. 23 strains expressed the greatest activity and obtained nanoparticles showed high stability. It was also noted that an increase in the initial concentration of copper ions in solution from 25 mg/l to 50 mg/l leads to an increase in the size of the resulting nanoparticles. Based on results of the UV-spectroscopy and AFM microscopy a database of microbial strains synthesizing copper nanoparticles was established, which may be used in future studies.

Keywords: Biosynthesis, Microorganisms, Copper Nanoparticles, *Fusarium, Pseudomonas.*

1. INTRODUCTION

One of the earliest studies on production of the metal nanoparticles by means of microorganisms (bacteria) was reported in 1964 [1]. But the electrochemical method was supposed as the most efficient for synthesis of large amounts of copper nanoparticles (CuNPs) within short period of time. Nevertheless, nowadays production of metal nanoparticles by living organisms has certain advantages compared to electrochemical one, such as environmental safety and conformity of size and shape [2, 3]. Metal nanoparticles possess different useful properties and are widely used in certain areas. There are many studies reported on antimicrobial activity of nanoparticles, including bacteriocidic activity of copper ions upon wide spectrum of microorganisms. Recently several reviews were published, which stipulated receipt of metal nanoparticles with use of microorganisms isolated from different ecological niches [4-8]. A great number of organisms are reported as producers of different metal nanoparticles: Ag, Au, Cd, Cu, Fe, Pb, Pd, Pt, Se, Zn, Zr and so on. Among those organisms are bacteria [5], fungi [9], yeasts [6], viruses [10], microalga [11], alga [12] and plant extracts [13].

Copper nanoparticles represent certain interest due to their antimicrobial, catalytic, electrical, optical and other properties. Varshney with colleagues have reported application of nonpathogenic strain of *Pseudomonas stutzeri* for the fast method of biological synthesis for

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production of 8-15 nm spherical CuNPs [14]. The innovative approach for synthesis of CuNPs by bacteria *Pseudomonas stutzeri*, earlier isolated from soil, resulted in receipt of 50-150 nm cubic shaped CuNPs [15]. Nowadays research activities on application of living organisms for production of metal nanoparticles, including copper, become extremely interesting. Thus, the aim of this work was screening of the culture collection of industrially important microorganisms of the Institute of Microbiology (Tashkent, Uzbekistan) for efficient producers of CuNPs.

2. MATERIALS AND METHODS

Strains of microorganisms preserved at the culture collection of industrially important microorganisms of the Institute of Microbiology (Tashkent, Uzbekistan) were used. Cultivation of fungi and bacteria was conducted on two times diluted Czapek-Dox, Mandels and beef extract peptone (BEP) nutrient media, respectively. Aliquots of copper sulfate 25 and 50 mg/l (for copper ions) were added to the cultural liquid of microorganisms. Mixture of cells and copper ions were incubated at the rotary shaker at 180 rpm and 28 °C for 24-72 h. Formation of the copper nanoparticles was visually monitored by change of the color of the solutions. Strains were selected according to their resistance towards different pollutants, including heavy metals, and ability to biosorption of metals as well, since the ability to produce metal nanoparticles is considered as a protective function of the microorganisms [16, 17]. UV-spectroscopic studies were conducted on a spectrophotometer SPECORD 210 (Germany) within range 190-1000 nm. The morphology of copper nanoparticles was studied using an atomic force microscope Agilent 5500 (USA) at the room temperature.

3. RESULTS AND DISCUSSION

Modern nanotechnologies are based on development of the reliable, non-toxic, ecologically safe technologies for the production of metal nanoparticles of the wide range of chemical composition, size, synchronized monodispersity, which is mainly possible to achieve by use of biological resources. Several reviews provided in details an information about isolation of the suitable microorganisms from different sources (soil, water, sewages, culture collections) [4, 8].

The culture collection of industrially important microorganisms of the Institute of Microbiology comprises a number of different strains both bacteria and fungi isolated from mines, flotation tails, industrial and household wastes and sewages, polluted soils and waters and so on. Based on analysis of available data several cultures were selected for the screening of ability to produce CuNPs. Filamentous fungi are more resistant towards mutations and possess ability to synthesize different nanoparticles. Selected fungal strains were preliminarily cultivated on nutrient medium containing copper salt. Analysis of selected collection cultures revealed among them several fungal strains capable to produce CuNPs (Table 1). The maximum activity among screened cultures was observed at *Penicillium* sp. 1, *Fusarium oxysporum* 1, and *Fusarium oxysporum* 2.

Table 1. Screening for CuNPs producing ability among selected fungal strains.

No.	Culture	Cultivation time (h)		
		24	48	72
1.	*Asperegillus niger*	-	-	-
2.	*Aspergillus terreus* 33	-	+	+
3.	*Aspergillus terreus* 4	-	-	+
4.	*Aspergillus terreus* 11	-	-	-
5.	*Aspergillus glaucus*	-	-	-
6.	*Aspergillus flavus*	-	-	-
7.	*Aspergillus versicolor*	-	-	+
8.	*Aspergillus albus*	-	-	-
9.	*Aspergillus oryzae*	+	+	+
10.	*Acremonium* sp.	-	+	+
11.	*Cladosporium cladosporioides*	-	-	-
12.	*Cladosporium* sp.	-	-	-
13.	*Alternaria* sp.	-	-	-
14.	*Penicillium* sp. 1	+	+	+
15.	*Trichoderma harzianum*	+	+	+
16.	*Alternaria pluriseptata*	+	+	+
17.	*Penicillium* sp. 2	-	+	+
18.	*Trichurus terrophilus*	+	-	-
19.	*Nocardia* sp.	-	-	-
20.	*Fusarium oxysporum* 1	+	+	+
21.	*Fusarium oxysporum* 2	+	+	+

Visual observation revealed that light blue color characteristic for the ions of Cu^{2+} disappears in the cultural liquid of *Penicillium* sp. and solution acquires light green-yellowish color. Formation of CuNPs by *Penicillium* sp. was determined by UV-spectroscopy methods at the initial concentration of copper ions 100 mg/l (Figure 1).

The absorption bands, differing by intensity, were formed at λ_{max} = 260 nm in both samples, regardless of the used nutrient medium. UV-spectrum of the strain cultivated on Czapek-Dox medium (sample 6) revealed a small shoulder at λ_{max} = 700 nm, testifying presence of Cu^{2+} traces; whereas cultivation on Mandels medium (sample 3) presented no such change.

Morphology of the test samples was studied by AFM method. It was established that systems medium+fungus+Cu^{2+} have both nano- and microstructure, the shape of nanoparticles is cubic. Sample 3 is characterized by formation of needle structures with diameter 800 nm and length 40 micron, which are produced as a result of aggregation of cubic nanoparticles with sized 300-400 nm (Figure 2).

There many reports are available on a wide variety of the fungi-producers of metal nanoparticles [3, 18-22]. Microscopic fungi are less susceptible to mutagenic factors like ions of metals, including heavy ones, and at the same time possess ability to synthesize different compounds, including metal nanoparticles. Nevertheless, there is no consensus on biological mechanism of the metal nanoparticles production. It is general assumption that there is no evidence that some specific type of protein or carbohydrate or lipid or any other molecule is the main factor responsible for production of the metal nanoparticles [23]. In these regards, apparently, proteins play fundamental role in production of CuNPs [2]. On the other hand, it was established that fungal enzymes affect the production of metal nanoparticles, and not only stability [3].

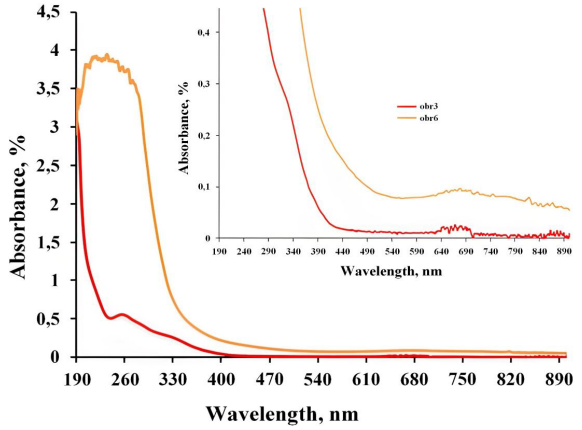
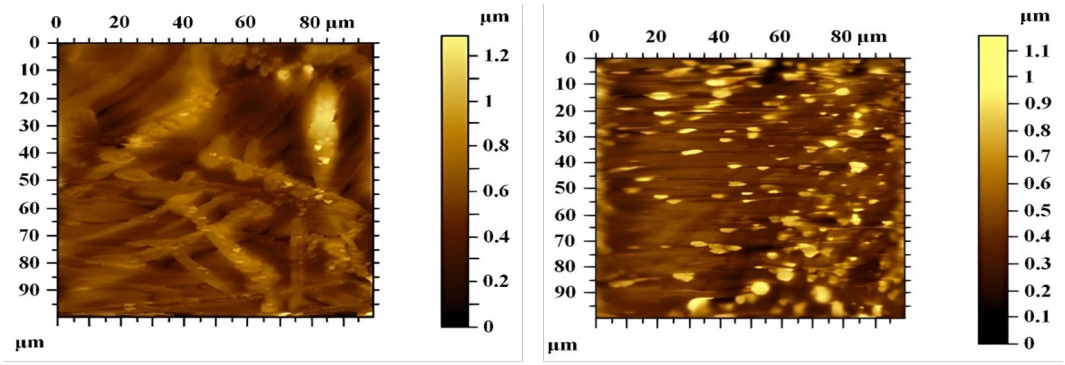


Fig. 1. UV-spectra of systems at different concentrations of CuNPs.

It is well known that bacteria may serve as perspective producers of metal nanoparticles [24-29]. Metal nanoparticles of the bacteria origin proposed for use in different fields of industry. Therefore, screening of the culture collection for potential bacterial producers of CuNPs was essential. Study on selected collection cultures of bacteria, earlier isolated from the polluted areas, established that maximum CuNPs producing activity was determined among strains related to *Pseudomonas, Bacillus* and *Acinetobacter* species (Table 2). *Acinetobacter* is not known as active producer of CuNPs, but it participates in formation of the other nanoparticles. Thus,



#3
d - 800 nm, length 40 nm

#6
100-800 nm

Fig. 2. Morphology of the test samples.

Acinetobacter was reported as an efficient producer of selenium nanoparticles when cell suspension and total cell protein of the strain *Acinetobacter* sp. SW30 was used [25]. In our case, capacity of this microorganisms to produce CuNPs was established (Table 2).

Based on preliminary results of the tested bacteria to synthesize nanoparticles, production of the CuNPs in dynamics of the process was studied on example of *Pseudomonas* species. It was established that all studied strains possess the ability to synthesize nanoparticles to one degree or another and the maximum synthesis is observed after 48-72 h (Table 3).

Strains *Pseudomonas stutzeri* and *Pseudomonas* sp. 23 revealed the best CuNPs synthesizing capacity, moreover nanoparticles synthesized by these strains expressed stability for up to a fortnight (Table 3). Its possible reason may be the formation of smaller nanoparticles and synthesis of compounds, which envelop nanoparticles preventing their aggregation and by this, probably, stabilizes them as well. Formation of the CuNPs in the cultural liquid of *Pseudomonas* strains was determined both visually and by UV-spectroscopy (Figures 3 and 4).

Table 2. Screening for CuNPs producing ability among selected bacterial strains.

No.	Culture	Cultivation time (h)		
		24	48	72
1.	*Pseudomonas putida*	+	+	-
2.	*Pseudomonas stutzeri*	+	2+	3+
3.	*Pseudomonas fluorescens*	+	+	+
4.	*Arthrobacter globiformis*	+	+	+
5.	*Bacillus megatherium*	+	2+	2+
6.	*Bacillus subtilis*	-	+	+
7.	*Bacillus* sp.	-	+	+
8.	*Acinetobacter* sp.	+	+	-+

Table 3. Screening of *Pseudomonas* strains for CuNPs synthesis.

Strain	Growth (days)					
	1	2	3	5	7	14
Pseudomonas stutzeri	+	3+	3+	3+	3+	3+
Pseudomonas putida 1	+	+	-	-	-	-
Pseudomonas sp. 23	+	2+	2+	2+	2+	+
Pseudomonas sp. R	+	+	+	+	+	+

UV-spectroscopy analysis revealed that, with an increase in the concentration of copper (II) ions, a bathochromic shift of the absorption wavelength is observed, which is directly proportional to the size of the nanoparticles. It was established that copper nanoparticles are observed in the form spherical particles, elongated particles are observed as well. At the same time, majority of studied microbial cultures, both bacteria and actynomycetes, produced insignificant quantities of copper nanoparticles.

It was established that nanoparticles were observed mostly after 24-48 h depending on certain microorganism and the highest synthesizing activity was observed after 48-72 h for the most of the cultures. More extended contact with copper salt causes aggregation of nanoparticles and precipitation, especially this is true in case of microscopic fungi.

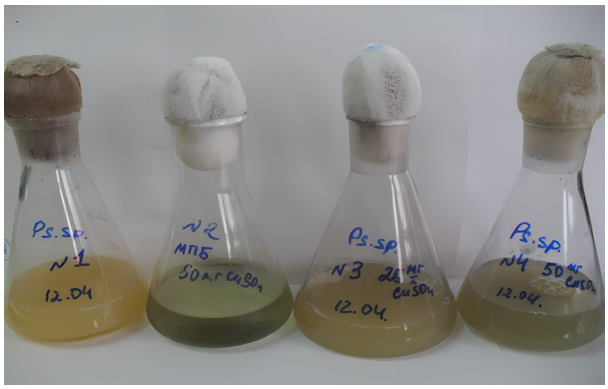


Fig. 3. Formation of CuNPs by strain *Pseudomonas* sp.: 1 – control (pure cultural liquid of the strain); 2 – BEP broth + $CuSO_4$; 3 – cultural liquid of *Pseudomonas* sp. with added 25 mg/l of Cu^{2+}; 4 – cultural liquid of *Pseudomonas* sp. with added 50 mg/l of Cu^{2+}.

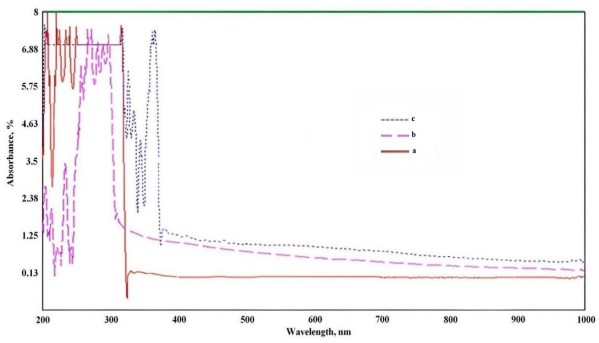


Fig. 4. UV-spectra of the systems with different amount of CuNPs: (a) beef extract peptone broth (BEPB); (b) BEPB+microorganism+Cu^{2+}(25 mg/l); (c) BEPB+microorganism+Cu^{2+}(50 mg/l).

4. CONCLUSIONS

From the present study we can conclude that strains *Fusarium oxysporum* and *Penicillium* sp 2, and *Pseudomonas* species were the most active producers of copper nanoparticles among all screened culture collection strains. Biogenic production of copper nanoparticles by bacteria and fungi presents certain scientific and applied interest. Based on results of the UV-spectroscopy and AFS microscopy a database of microbial strains synthesizing copper nanoparticles was established, which may be used in future studies.

5. CONFLICT OF INTEREST

The authors declear no conflict of interest.

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Research Article

Anti-bacterial and Anti-biofilm Effect of Curcumin-Ag Nanoparticles against *Pseudomonas aeruginosa* Isolated from Iraqi Burn Patients Infections

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Abstract: The increasing emergence of multidrug-resistant bacteria, which are the cause of wound infections, constitutes a major health problem, and because of their ability to produce biofilms. The main objective of the present study is to evaluate the antibacterial and antibiofilm activity of curcumin-Ag nanoparticles. According to Scanning Electron Microscopy (SEM) and X-Ray Diffraction analysis (XRD), the nanoparticles appeared in spherical shapes and sizes of 47.98 - 58.80 nm. From UV-Visible spectrum a high-intensity absorption peak around 450 nm called the spectral plasmonic region (SPR), is observed for curcumin-Ag. To examine the antibacterial activity, the agar-well diffusion method was performed. Minimum inhibitory concentrations (MIC) of curcumin-Ag nanoparticles and gentamicin were used to evaluate the antibacterial activity against resistant *Pseudomonas aeruginosa*. The results indicate that nano-curcumin possesses material anti-bacterial activity against all *Ps. aeruginosa* isolates disparity with control, and the anti-bacterial activity of nano-curcumin at 256 µg/ml was significantly higher than 128 µg/ml, according to earlier research, curcumin nanoparticles break down bacterial cell walls, and when this happens, the bacteria lyse and die. Antibiotic susceptibility testing was performed on Piperacillin (70%), Imipenem (53.33%), Colstine (40%), Gentamycin (0%), and Ceftaroline (CFT) (30%). Significant antibacterial action of curcumin NPs was observed against the most biofilm-producing *Ps. aeruginosa* isolates.

Keywords: Antimicrobial Activity, Antibiofilm, Burn, Curcumin Silver Nanoparticles, Characterization, *Pseudomonas aeruginosa.*

1. INTRODUCTION

Burn patients are more susceptible to hospital-associated infections, when the skin is burned, it disrupts the physiological function of the immune system and destroys the skin's protection from infection [1]. *Ps. aeruginosa* is a Gram stain-negative (-) bacillus. It is aerobic and causes unscrupulous or nosocomial contagion in burn patients, wound infections, cystic fibrosis, and folks who suffer from immunodeficiency [2]. Furthermore, the development of biofilms, which are of a vital and critical virulence factor that improves the survivability of bacteria in these settings, what allows bacteria to survive in harsh environments, such as drought or the presence of disinfectants [3]. Formation of biofilms is one of the reasons aimed at antibiotic resistance, and also serve as a partition between antibiotics and bacterial cells or immune responses [4]. Furthermore, in the case of burn injuries, eschar formation prevents access of host immune cells to the infected area and systemic administration of antibiotics [5]. Most are susceptible to anti-pseudomonal antibiotics, but the difficulty of eradication adds to the problem of burn management. Burn injuries are chronic and incurable diseases that are difficult to treat due to the evolution and changes in the antimicrobial properties of the pathogens involved. Curcumin is a major phytochemical resulting from C. longa (Zingiberaceae), which is known as turmeric, a naturally occurring yellow

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pigment [6]. When combined with other drugs, this compound has anticancer and antioxidant properties [7, 8]. Extensive research conducted over the past 50 years indicates that curcumin has powerful antioxidant, anti-inflammatory, anti-tumor, anti-HIV, and antimicrobial effects [9-12]. Nanoparticles have singular properties in electronic, magnetic, and chemical energizing. Nanoparticles are also characterized via loudly stability, lack of reaction, biocompatibility, and comparatively shortage of toxicity. That is why these are widely applied in numerous domains of biomedicine, such as industry, gene delivery, etc. Certain mineral nanoparticles also have antiviral, antibacterial, antifungal, and antitumor possessions [13]. In general, there are three requisite methods for building nanoparticles: Chemical methods, Physical methods and Biological methods [14].

Silver nanoparticles (Ag-Nps) are the most studied and most widely used of all nanoparticles. Ag-Nps are now regarded as next-generation antibiotics. This is because these are highly effective in suppressing microorganisms. Ag-Nps are currently the leading nanoparticles among all commercialized nanomaterials. Research into their use as antibacterial agents has intensified over the years due to their low toxicity compared to other nanoparticles. Adherence and penetration of Ag-Nps onto microbial membrane surfaces is typically the first step in their cytotoxic mechanism [15]. One of the drawbacks of the synthesis of chemical and physical methods is that these are comparatively expensive, adding to chemical methods involving the use of elements and compounds. It has poisonous and risky effects on researchers, and some hazards are graver than the environment or neighborhood in which it is located. Lives in it because several physical and chemical mechanisms lead to the generation of nanoparticles that are not suitable. You can't control the shape, size, and purity you need, so you have to find a way safer, more accurate and cheaper [16]. Alternatively, a method known as the green method produces a more homogenous material Fewer defects resulting from nanoparticle formation by microorganisms such as bacteria and fungi or algae, plants, or plant extracts, many characteristics of organisms such as pathways Biochemical, enzymatic activity, stage of cell growth, and optimal response are considered Defines a selection of objects or their extracts for building nanoparticles [17]. Researchers have looked into the special qualities of synthesized green nanoparticles [18]. As a result, nanoparticles are increasingly being used in medicine development, coatings, and food packaging [19, 20]. It has been demonstrated that using nanoparticles as carriers in drug delivery systems increases bioavailability, stability, pharmacological, and solubility effect while preventing cytotoxicity to healthy cells, chemical and physical dissolution, and excessive dose requirements. Because of their numerous potential applications as antibacterial, antifungal, antioxidant, anticancer, and anti-inflammatory medicines, silver nanoparticles (Ag-Nps) have piqued the interest of researchers and scientists [21]. Ag-Nps can withstand a broad variety of temperatures and are far less volatile than other nanoparticles [22], that can limit microbial development after initially coming into contact with bacteria [23]. For the management of *Ps. aeruginosa* infections in those who already have them, new medications and other therapies must be created right away when traditional antibiotics become ineffective. New antibiotics with distinct modes of action, novel dosage techniques, and resistance to bacterial enzyme changes have all been the subject of recent research [24, 25]. The aim of this research is to explore for substitutes, such as nanomaterials, which have emerged as a promising alternative to antibiotics in recent years, such as the antibacterial and antibiofilm activity of curcumin-Ag nanoparticles. We hope that the present research findings will address the growing problem of multidrug-resistant bacteria, which cause wound infections and are a major health concern due to their capacity to form biofilms.

2. MATERIALS AND METHODS

2.1. Synthesis of Curcumin Silver Nanoparticles

To get a final concentration of 10.6 μM, a tiny amount of an ethanolic solution of curcumin (10.6 mM, 1 ml) was first dispersed in 1 L of ultrapure water. The pH of the solution was brought to 8.5 - 9 by a tiny amount of 1 M NaOH. A little amount of AgNO3 (1 mM, 1 ml) solution was added to 99 ml of boiling curcumin solution at a moderate stirring speed (350 rpm) in order to produce particles. For 48 hours, the resultant solution was dialyzed against 10 mM borax buffer, with a dialysis medium change occurring every 24 hours [26]. After making certain adjustments to the green color

compositing procedure, we carefully examined the results using the Bettini protocol to obtain the naturally occurring silver-capped NPs compound, also known as curcumin-Ag [26]. The purpose of the synthesis was to address the issue of lengthy contact times. We used a reaction flask, instead of an oven with adequate ventilation, to conduct the Curcumin-NP reaction. The Bettini method called for carefully adjusting the pH and raising the reaction temperature from 90 to 100 °C [27].

2.2. Characterization of Curcuma Silver Nanoparticles (Cur-Ag-Nps)

X-ray diffraction (XRD) is being used to characterize the phase identification, structure of crystals, composition and physical characteristics of curcumin nanoparticles. The nanomaterial is deposited on a piece of glass to measure it by (a Lab XRD Shimadzu XRD-6000) in Iran. A high-precision vertical goniometer built-in X-ray diffraction is dependent on monochromatic X-ray constructive interference and a crystalline sample. The KBr technique was used to obtain the Fourier transform infrared spectrum (FTIR). The material is pressed into a disc after being combined with KBr in a (1:1) molar ratio. A little quantity of moisture was put on a glass slide to dry before measurement, and measurements were run in the wavenumber range of 600 to 4000 cm^{-1} to evaluate and determine the functional groups present in the produced curcumin nanoparticles. It should be stored in a dust-free environment overnight before being measured. Field Emission Scanning Electron Microscopic (FESEM) study was conducted using VEGA 3 (TESCAN, Czech Republic) SEM machine. SEM was used to visualize the morphology and nanoparticle grain size of tested samples. Thin films of curcumin nanoparticles were prepared on the cover slide grid by reducing the amount of solution on the cover slide and then allowed to dry at room temperature before being visualized under SEM. The hydrodynamic diameter of the curcuma silver nanoparticles (Cur-Ag-Nps) and their possibility were gauge through the Dynamic light scattering (DLS) technique, whereas the TEM pictures obtained with a JEOL 1200 EX TEM running at a 120 KV acceleration voltage were statistically analyzed to determine the diameter of the particles' silver center. UV-analysis using Spectral ax was used to determine the existence and make-up of the curcuma shell encircling the particles [28]. These characterizations were carried out at the College of Science, Mustansiriyah University, Iraq.

2.3. Sources of Clinical Bacteria Isolates

Thirty (30) bacterial isolates belonging to *Pseudomonas aeruginosa* were isolated from people with burns and lying in bed in Yarmouk Hospital in the city of Baghdad. The diagnosis of the bacteria as *Pseudomonas aeruginosa* was confirmed using the Vitek 2 device.

2.4. Testing of Antibiotic Susceptibility

Testing susceptibility of antibiotic was executed by the Kirby power disk diffusion method; this method is described in details elsewhere [29]. Antibiotic tablets Ceftaroline (CFT) (30), Colstine (CT) (10), Gentamycin (GN) (10), Imipenem (IMP) (10), and Piperacillin (PRL) (10) were used for antibiotic susceptibility testing (AST). According to Magiorakos *et al*. [30], isolates that show resistance to three or more distinct types of antipseudomonal drugs are classified as multidrug resistant (MDR). In this study, *Escherichia coli* ATCC strain 25922 was used for quality control.

2.5. Biofilm Formation Assay

Two diverse methods, Congo red agar (CRA) and microtiter plate assay (MPA), were used to detect biofilms in each isolate. Biofilm production by all isolates was detected using the CRA method, as reported previously [31, 32]. All isolates were grown on Congo red agar (CRA) and examined for biofilm production. For the MPA method, as stated by Zhang *et al*. [33], the identified and purified *Pseudomonas aeruginosa* bacterial isolates were transferred to Vortex to mix and homogenize them well, then they were placed in a liquid medium (Luria-Bertani broth) for the purpose of culturing them for 18 hours at 37 °C. 96-well microtiter plates were used, and the bacterial isolates were transferred to these plates. Then the microtiter plates were transferred to the incubator at a temperature of 37 °C for 18 hours. A spectrophotometer was used at a wavelength of 540 nm. The range of the optical density was 0.56 to 0.64. Following the incubation time, 96-well microtiter plates were filled with 25 ml of 1% crystal violet per well. After letting them sit at room temperature for fifteen (15) minutes, thoroughly wash each with 200 milliliters of sterile

buffer, PBS. A UV spectrophotometer (Shimadzu, Japan) was used to detect the absorbance at 540 nm after crystal violet was dissolved in glacial acetic acid to gauge the degree of biofilm formation. Using crystal violet as a control to wells that are exposed to a medium devoid of germs, every assay was run in triplicate [34].

2.6. Determination of Nano-Curcumin-Ag and Gentamicin MIC and Sub MIC

Depending on the micro dilution method described by Elshik *et al*. [35] different concentrations of gentamicin and Nano-curcumin-Ag (1-1024 µg/ml) melted in Muller-Hinton broth (MHB) were prepared from a stock solution in a 96-well polystyrene microtiter plate. The bacterial *P. aeruginosa* suspension (10 µl) adjusted to 0.5 McFarland standard was additional to each well except for the monitoring of negative wells contained gentamicin culture was considered as a positive well, the microtiter plate was incubated overnight at 37 °C. Resazurin (0.015%) was additional to all wells (30 µl per well) and incubated for an additional 2-4 h to observe any color change after 24 h at 37 °C, after the incubation period was through columns with no color change the blue resazurin color stayed the same after the incubation period was through, columns with no color change (the blue resazurin color stayed the same) given a score the MIC value.

2.7. Antibiofilm Activity of Nano-Curcumin-Ag and Gentamicin Sub-MIC Against *Ps. aeruginosa* Isolates

Biofilm development was investigated using the same procedure as described previously [36]. Each well received 180 µl of nano-curcumin-treated BHIB and sub-MIC gentamicin, 20 µl of *Ps. aeruginosa* suspension, and a control of 0.5 MacFarland after sterile tryptic soy broth with 2% sucrose was prepared. Following incubation, the medium was taken out of the wells, rinsed three times with sterile buffer (PBS) to get rid of any remaining *Ps. aeruginosa* cells, and allowed to dry for fifteen (15) minutes at room temperature. After adding 200 µl of 0.1% purple crystals, waited for 20 minutes. The stained wells were rinsed three times with PBS (PH 7.2) and allowed to dry at room temperature for 15 minutes in order to eliminate any remaining dye. The optical density was then measured after 200 µl of 95% ethanol was added to each well, by using ELISA reader at 630 nm.

2.8. Antibacterial Activity of Nano-Curcumi-Ag Against *Ps. aeruginosa* Isolates

Used the agar well diffusion method to detection antibacterial activity of nano-curcumin against *Ps. aeruginosa* at concentrations of 128 µg/ml and 256 µg/ml, according to the procedure described by Kunwa *et al*. [37].

2.9. Statistical Analysis

The obtained data were subjected by analysis of variance (ANOVA) test to compare the means of various groups with each other. LSD test was used to calculate the significant differences between tested mean, the letters (A, B, C, and D) LSD for rows represented the levels of significant, highly significant start from the letter (A) and decreasing with the last one. Similar letters mean there are no significant differences between tested mean. Results were expressed as mean ± SD and values of $p > 0.05$ were considered statically non-significant, while $p < 0.05$, < 0.01, and 0.001 were considered significantly different, highly significantly different respectively. The statistical analysis was carried out by SPSS (v 20).

3. RESULTS AND DISCUSSION

3.1. Synthesis of Curcumin Silver Nanoparticles

Color change of the resulting colloidal material is shown in Figure 1; this solution confirmed the formation of Cur-Ag-Nps, which was subsequently confirmed by UV-visible spectroscopy. The results were similar to a previous study by Khan *et al*. [18].

3.2. Characterization

Following synthesis, it was eliminated by

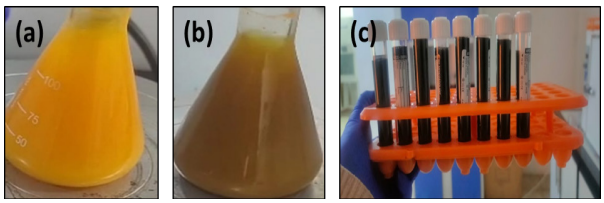

Fig. 1. Synthesis of Curcumin Silver Nanoparticles, (a) Curcumin solution, (b) Curcumin & AgNO3, and (c) Cur-Ag-Nps.

hemodialysis, where ultrapure water for borax buffers was used for NP, after the pH was decreased and the reaction residue was removed. To make Bettini's synthesis mixes as biocompatible as possible without sacrificing particle stability, we used a buffer (borax). The buffer was concentrated to a level that eliminated the possibility of osmotic stress-induced, which can harm to human cells during exposure. According to the nanoparticles' X-ray diffraction study results (Figure 2). The diffraction peaks at angles (2θ) of 38.2359°, 44.3076°, 64.5258°, and 77.4274°can be attributed to crystalline lattices (111), (200), (220), and (311), respectively of the face-centered cubic crystalline structure that accorded with the standard silver card values (JCPDS No.87-0720). Its purity and highly crystalline quality are indicated by the highest peak, which is found at 38.2359°. The XRD profiles of curcumin-loaded Ag-Nps, as shown in Figure 2, affirming its highly crystalline nature [38]. The XRD pattern of curcumin-loaded Ag-Nps also displayed characteristic peaks within the 2θ confirming the successful conjugation of curcumin onto the synthesized nanoparticles [39].

The ground sample's SEM revealed that the particles were synthetic, with an average diameter of 47.98 - 58.80 nm (Figure 3). The dried curcumin nanopowder was discovered to have good chemical and physical stability. Similar findings from earlier studies have indicated that reducing the active component's particle size to nanoparticle size improves its solubility and bioavailability [13]. It should be noted that these results agree with the previous XRD data [38, 39], that were obtained using the same preparation process. Gevorgyan *et al*. [40] also prepared nanoparticles using various techniques for cardiovascular complications.

FTIR is a powerful technique that works on the principle that ligand clusters vibrate at different frequencies. It can be used to detect functional

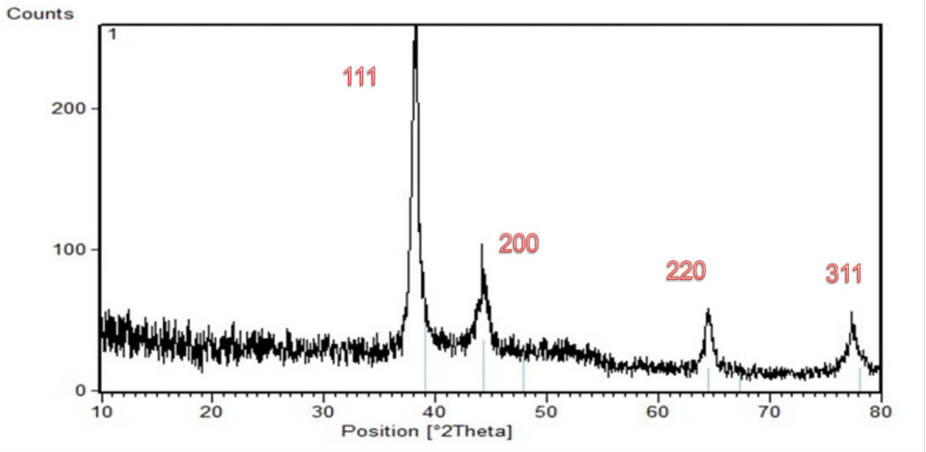

Fig. 2. Powder X-ray diffraction (XRD) of curcumin NPs.

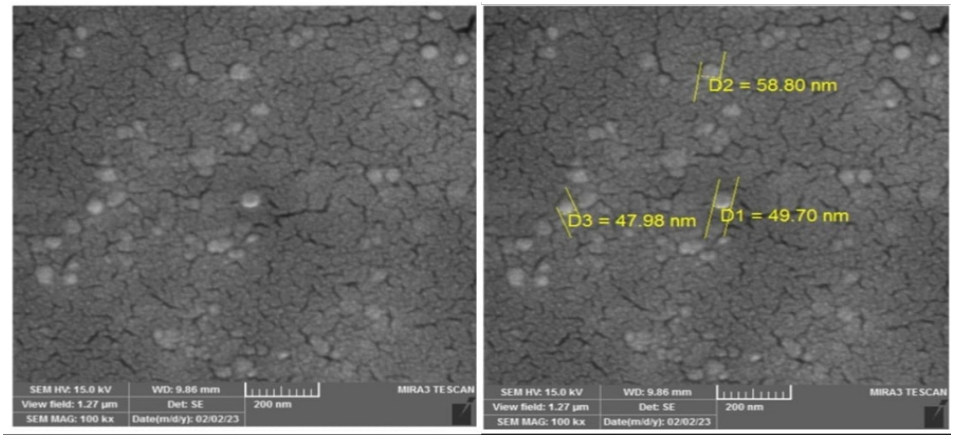

Fig. 3. Scanning Electron Microscopy SEM Analysis of Curcumin nano particles.

groups and characterize covalent bonds. FTIR spectrum of nano-curcumin is shown in Figure 4, where peaks are observed at 2594.04, 1842.22, 1264.04, and 927.61 cm^{-1}. The broad stretch at 3435.91 cm^{-1} represents the stretching vibration of the frequency (stretching) of the O-H bond with the hydrogen bond present in curcumin nanoparticles. The sharp peak at 2594.04 cm^{-1} indicates O-H stretching bonds of the functional group, carboxylic acid. The sharp peak at 2076.41 cm^{-1} indicates CH stretching of methylene groups. The sharp peak at 1842.22 cm^{-1} indicates C=O stretch bonds of functional group anhydride. The sharp peak at 1633.84 cm^{-1} is the stretching vibration of C=O and C=C double bonds. The sharp peaks at 1264.04 cm^{-1} and 1025.98 cm^{-1} C-N bonds of functional group amines. The sharp peak at 927.61 cm^{-1} C-O bonds of functional group anhydrides. The sharp peak at 703.57 cm^{-1} represents the C-Cl bonds of functional group alkylhalides. These results are consistent with the research of Joly and Latha [41], who showed that nanocurmin has strong intensity and sharp peaks.

Figure 5 shows the UV-Visible spectrum of curcumin silver nanoparticles (curcumin-Ag-Nps) in high-purity water. A high-intensity absorption peak around 450 nm called the spectral plasmonic region (SPR), is observed for curcumin NPs. These results are consistent with the previous studies [28, 41. The UV-visible spectroscopy can be used to verify nanoparticle constancy, or the stability of nanoparticles in liquid. When the particles lose their stability, or become destabilized (due to the exhaustion of stable nanoparticles), the intensity of the initial extinction value decreases.

Several images were taken using delivery transmission electron microscopy (TEM) in order to examine the size of Cur-Ag-Nps and calculate their average silver core diameter (Figure 6 (a, b). After that, the images were processed using image analysis software (ImageJ) to determine the diameter of each particle in each picture. The TEM images of curcumin-Ag nanoparticles reveal that the nanoparticles are spherical in shape, and the average size is about 50 nm.

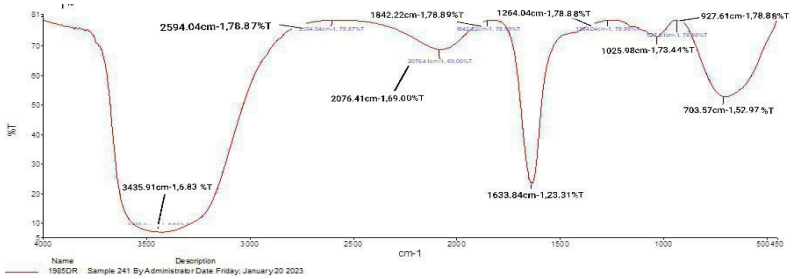

Fig. 4. Fourier transform infrared (FTIR) for identification of encase compounds in curcumin-Ag-NPs.

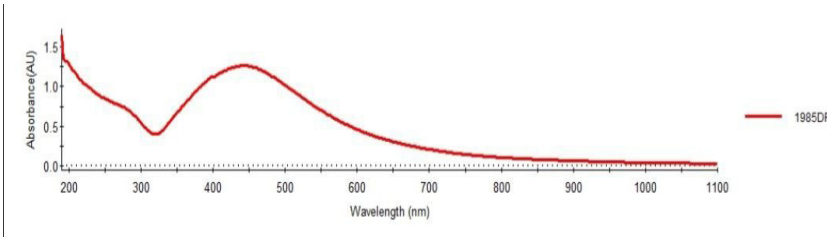

Fig. 5. UV–Visible spectrum of Curcumin NPs.

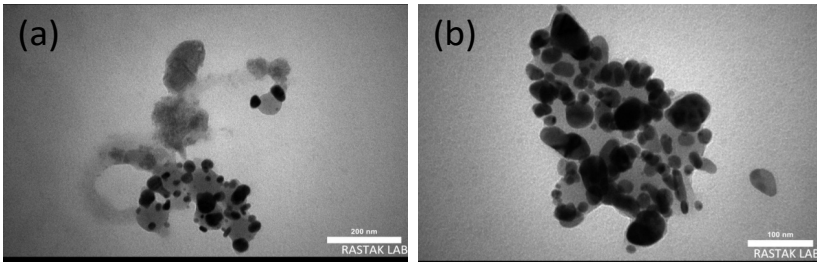

Fig. 6. Transmission electron microscopy (TEM) images of Cur-Ag-Nps. (a) 7500x magnification and (b) 10,000x magnification.

Figure 7 shows the DLS investigation of Cur-Ag-Nps dispersions using dynamic light scattering. The curcumin shell and the amount of water bonded to it have a direct impact on the electrostatic charge since DLS assesses the molecule's hydrodynamic diameter [43]. Large particles scatter more light than small particles, hence DLS tends to overestimate them [44]. The material's electron density in relation to the supporting carbon lattice determines how different the TEM image is. Because of this, the molecule's curcumin layer is thin and nearly impossible to photograph using TEM imaging because of its poor contrast [45]. Similar findings were found in earlier research, which demonstrated that the active element's efficacy, solubility, and bioavailability are increased when its particle size is reduced to nanoparticle size [46].

3.3. Antibiotic Susceptibility Test for *P. aeruginosa* Isolates

The antibiotic susceptibility test (AST) was managed for the isolates of bacteria *Ps. aeruginosa* using the disc diffusion process with five antibiotics from dissimilar classes. The results of antibiotic susceptibility of *Ps. aeruginosa* showed that 70% of isolates were resistant to Piperacillin follow by 53.33% resistant to Imipenem (FEP), 40% were resistant to Colstine, 30% were resistant to Ceftaroline, and 0% *Ps. aeruginosa* isolates were resistant to Gentamycin, these results are shown in Figure 8. It is unclear from the results if gentamicin is the most effective medication when compared to other antibiotics; this could be because the isolates have various antibiotic resistance mechanisms. Various antibiotics, particularly those in the minoglycoside class, cause *Pseudomonas aeruginosa to* develop resistance to pharmacological classes known as beta-lactams and quinolones [47]. Another study by Akingbade *et al*. [48] showed that *Pseudomonas aeruginosa* contains a large number of resistance genes, including extended resistance genes. A spectrum of β-lactamases (ESBLs), aminoglycoside-modifying enzymes (AMEs), and β-lactamases shows that they can rapidly mutate and acquire drug resistance to adapt to the environment and spread resistant bacteria [49]. The incidence of antibiotics is increasing year by year [50].

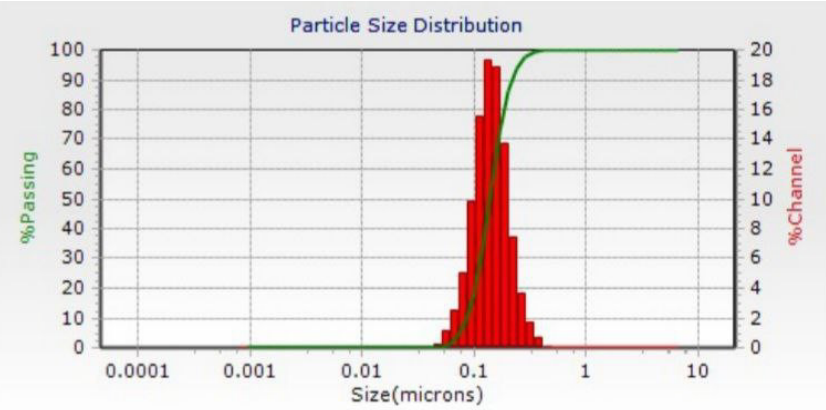

Fig. 7. Dynamic light scattering DLS analysis of "curcumin silver nanoparticles" "Cur-Ag-NPs" dispersions.

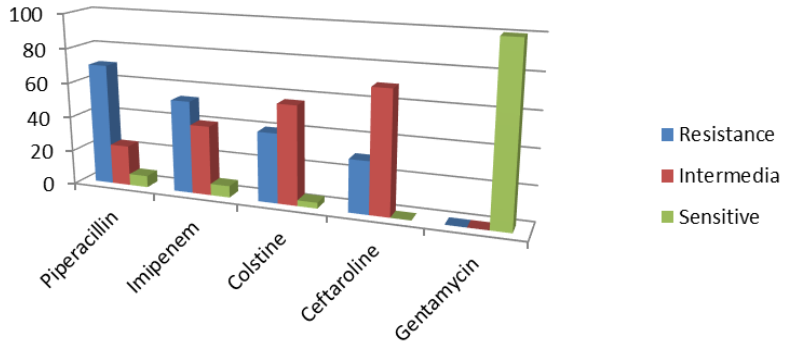

Fig. 8. Results of antibiotics susceptibility test to *Pseudomonas aeruginosa*.

3.4. Biofilm Formation of *Ps. aeruginosa*

Ps. aeruginosa have probable biofilm formation capability on Congo Red Agar (CRA). The colony color of isolates changes to black, an indication of the productivity of biofilms (Figure 9), and biofilm formation capability on the microtiter plate (Figure 10). The obtained results were labeled into 4 groups (non-biofilm production, weak, moderate, and strong) based on threshold values. Based on the criteria listed in Table 1 of the 30 Ps. In the *Ps. aeruginosa* isolates in this study, 3 strains formed weak biofilms, 11 strains formed moderate biofilms, and 16 strains were shown to have formed a strong biofilm. Cutoff values for *Ps. aeruginosa* isolates are summarized in Table 1. The result showed only 53.3% of *P. aeruginosa* isolates were strong producers for biofilm, while 36.7% and 10% of the isolates were moderate and weak producers for biofilm, respectively. This was mentioned in a previous study of 100% of *Ps. aeruginosa* isolates are biofilm productive [51], which supports our findings. Numerous studies have demonstrated that *Pseudomonas aeruginosa* MDR produces more biofilms than other harmful bacteria, demonstrating the synergistic influence of biofilm formation and antibiotic resistance.

Fig. 9. Biofilm formation by *Ps. aeruginosa* on Congo Red Agar, it contains (brain heart infusion broth, sucrose, agar and Congo red solution).

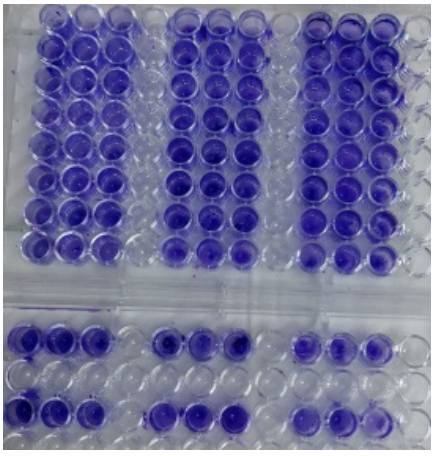

Fig. 10. Biofilm formation by *Ps. aeruginosa* on microtiter plate.

3.5. Determination of Nano-Curcumin-Ag and Gentamicin MIC and Sub MIC

The four isolates of *Ps. aeruginosa* that produced the most biofilm (No. 12, No. 14, No. 25, and No. 26) were examined for their susceptibility to gentamicin and nano-curcumin. It was observed that isolates varied in their susceptibility to gentamicin and nano-curcumin. Gentamicin was employed as a control since clinical isolates were responsive to it (Table 2).

3.6. The Antibiofilm Activity of Nano-Curcumin and Gentamicin Sub MIC Against *Ps. aeruginosa* Isolates

Nano-curcumin shows a significant antibacterial film activity against the tested isolates of *Ps. aeruginosa* no. 6 biofilm (OD 1.732 ± 0.236, reduced to 0.216 ± 0.065) and no. 12 biofilm (OD 0.987 ± 0.152, reduced to 0.194 ± 0.145). In contrast, the OD of the no. 15 biofilm decreased to 0.309 ± 0.109 from 1.589 ± 0.178. In vitro, nano-curcumin exhibited more anti-biofilm action than gentamicin, as illustrated in Figure 11. Using treatments with minimum inhibitory concentrations (MIC values), such as gentamicin and nano-curcumin, is one way to eliminate biofilms formed by previous isolates [51]. It has been reported that the minimum inhibitory concentrations (MIC) of gentamicin and amikacin are utilized to reduce the growth of biofilms on plastic sheets. Several studies have shown that some pure substances, like bacteriocins and enzymes, have potent anti-microbial and anti-biofilm qualities that outperform gentamicin in their ability to fight a range of pathogens in vitro. The theories include mechanisms that function against bacteria, including cytoplasmic membrane defects, DNA degradation, bacterial cell wall defects, and interference with bacterial cell division [50, 51]. A study by Bassetti *et al.* [52] found that *Pseudomonas aeruginosa* infections are treated with aminoglycosides like tobramycin, gentamicin, and amikacin. The minimum inhibitory concentration (MIC) is used to evaluate the antimicrobial potency of novel compounds or

Table 1. Measured Optical Density (O.D.) of Biofilm Formation Capability of *Pseudomonas aeruginosa* Isolates.

Isolate *pseudomonas aeruginosa* No.	O.D.	Isolate *Pseudomonas aeruginosa* No.	O.D.
Pseudomonas aeruginosa 1	0.253	*Pseudomonas aeruginosa 16*	0.186
Pseudomonas aeruginosa 2	0.913	*Pseudomonas aeruginosa 17*	1.23
Pseudomonas aeruginosa 3	0.183	*Pseudomonas aeruginosa 18*	1.09
Pseudomonas aeruginosa 4	0.860	*Pseudomonas aeruginosa 19*	0.96
Pseudomonas aeruginosa 5	1.016	*Pseudomonas aeruginosa 20*	1.08
Pseudomonas aeruginosa 6	0.290	*Pseudomonas aeruginosa 21*	0.053
Pseudomonas aeruginosa 7	0.276	*Pseudomonas aeruginosa 22*	0.048
Pseudomonas aeruginosa 8	0.172	*Pseudomonas aeruginosa 23*	0.153
Pseudomonas aeruginosa 9	0.158	*Pseudomonas aeruginosa 24*	0.96
Pseudomonas aeruginosa 10	0.073	*Pseudomonas aeruginosa 25*	1.49
Pseudomonas aeruginosa 11	1.15	*Pseudomonas aeruginosa 26*	1.92
Pseudomonas aeruginosa 12	1.4	*Pseudomonas aeruginosa 27*	0.162
Pseudomonas aeruginosa 13	0.280	*Pseudomonas aeruginosa 28*	1.4
Pseudomonas aeruginosa 14	1.76	*Pseudomonas aeruginosa 29*	1.06
Pseudomonas aeruginosa 15	o.237	*Pseudomonas aeruginosa 30*	0.288

Table 2. Minimum inhibitory concentration (MIC) and sub MIC of Gentamicin and Nano-curcumin for *Pseudomonas aeruginosa.*

Pseudomonas aeruginosa isolates	**Sub MIC of Gentamicin mg/ml**	**MIC of Gentamicin mg/ml**	**Sub MIC of Nano-curcumin mg/ml**	**MIC of Nano-curcumin mg/ml**
Pseudomonas aeruginosa 12	512	1024	128	256
Pseudomonas aeruginosa 14	128	256	64	128
Pseudomonas aeruginosa 25	512	1024	128	256
Pseudomonas aeruginosa 26	256	512	512	1024

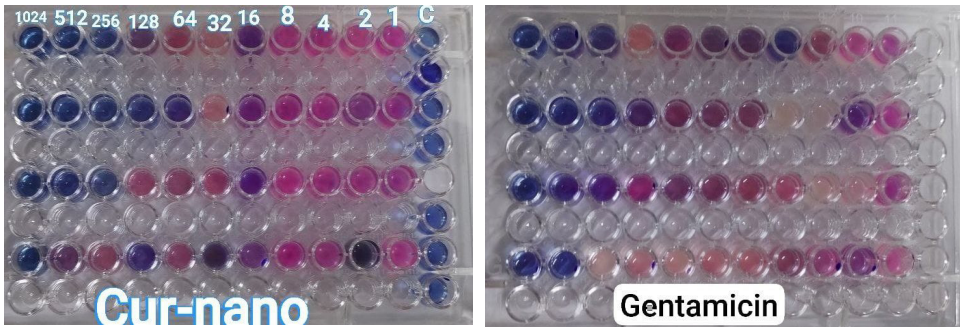

Fig. 11. The sub minimum inhibitory concentrations (MIC) of Gentamicin and Nano-curcumin against *Pseudomonas aeruginosa.*

extracts by determining how efficiently they reduce antibacterial concentrations. Antibiotics with lower MICs are more effective [53]. Conveyed uses sub-MIC doses of gentamicin and amikacin to decrease the formation of biofilms on plastic components. Several investigations have shown that some pure compounds, like enzymes and bacteriocins, exhibit strong antibacterial and antibiofilm action and are more effective than gentamicin against a range of infections in vitro. These investigations have also suggested a number of potential antibacterial processes, including disruption of the cytoplasmic

membrane and cell wall, impairment of cell division, and DNA destruction [51]. The minimum inhibitory concentration (MIC) of an antimicrobial agent is the lowest concentration (µg/ml) that completely stops the detectable development of a test strain of a bacterium under closely watched laboratory conditions. will be finished. best prediction for the clinical outcome. Antibiotics are used more precisely and effectively [54]. Alternatively, by altering the expression levels of bacterial virulence genes, for example, a minimum inhibitory concentration (sub-MIC) below sub-MIC can influence bacterial pathogenesis [55]. Different phenotypes are caused by gender. According to a study by [56], there are a number of reasons why bacteria are more likely to be encountered through the minimum inhibitory concentration (sub-MIC) of antibiotics, including restricted access to medications and the use of low-dose antibiotics as a preventative measure. The biofilms of earlier isolates were treated with gentamicin and nano-curcumin at minimum inhibitory concentrations (MIC values) [51].

3.7. Antibacterial Activity of Nano-curcumin on *Ps. aeruginosa*

Table 3. shows the antibacterial activity of nano-curcumin against *Ps. aeruginosa* solates at concentrations of 32, 64, 128 and 256 µg/ml. The results indicate that nano-curcumin possesses material anti-bacterial activity against all *Ps. aeruginosa* isolates disparity with control, and the anti-bacterial activity of nano-curcumin at 256 µg/ml was significantly higher than 128 µg/ml. The sensitivity of *Pseudomonas aeruginosa* to nano-curcumin was measured by a micro-dilution broth assay (Figure 11). Nano-curcumin solutions were prepared in distilled water and measured by the well diffusion method. The inhibition zone was measured at concentrations of 128 µg/ml and 256 µg/ml. Nano-curcumin MICs for four strains of *Pseudomonas aeruginosa* were 128 µg/ml and 256 µg/ml (Figure 12), due to the fact that curcumin nanospheres dissolve more readily [57]. Cur-NPs show strong antibacterial action against *Ps. aeruginosa*, and the outcomes were comparable to those of *Ps. aeruginosa* [58]. Our findings showed that nanoparticles had broad-spectrum inhibitory effects on isolates of *Ps. aeruginosa*. According to an earlier research, curcumin nanoparticles break down bacterial cell walls, and when this happens, the bacteria lyse and die [59]. A mixture of silver nanoparticles and curcumin NPs at 100 µg/ml inhibits 50% of bacterial biofilms, according to another study by Loo *et al*. [60]. Curcumin-coated nanoparticles were found to suppress growth in vitro, according to Krausz *et al*. [61]. Additionally, Alsammarraie *et al*. [62] demonstrated that Ag-Nps

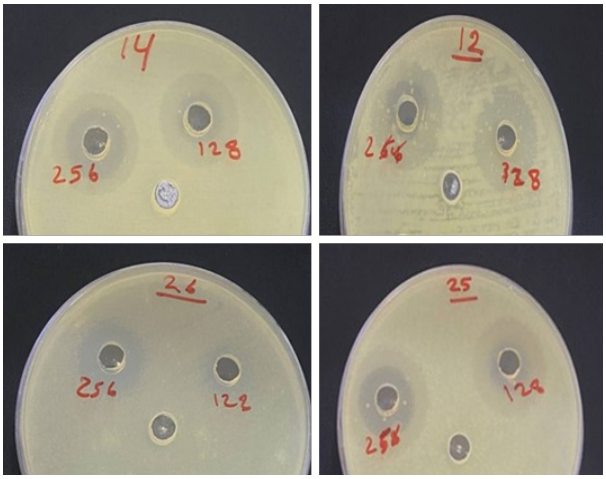

Fig. 12. Antibacterial activity of nano-curcumin against *Pseudomonas aeruginosa* isolates.

Table 3. Antibacterial activity of Curcumin-Ag-Nps on *Pseudomonas aeruginosa* in-vitro.

Treatment	(Mean ± SD) The inhibition zone diameter (mm) concentration µg/ml, N = 10				P value
	32	64	128	256	
Curcumin nano	13.3±5.9a	A 15.3±5.7a	22.3±1.96b	24.3±1b	<0.01 sig
P value	<0.01	<0.01	<0.01	<0.01	

LSD test was used to calculate the significant differences between tested mean, the letters (A, B and C) LSD for rows represented the levels of significant, highly. Similar letters mean there are no significant differences between tested mean. (Sig: significantly).

* Significant differences compared with enzyme and curcumin nano $p < 0.01$.

had antimicrobial properties. Ag-Nps anti-cancer and antioxidant properties have been demonstrated previously [63, 64]. Curcumin's significant action against MRSA and *Staphylococcus aureus* with MLSB resistance phenotype was established in a study by Górski *et al*. [65], with comparable MIC values at a median level of 0.046 mg/ml. Curcumin has been shown to have antibacterial properties against Methicillin-Resistant *Staphylococcus aureus (MRSA*) and *Staphylococcus aureus* [66]. Through its quorum sensing regulation mechanism, curcumin functions as an antibiofilm by inhibiting host receptor attachment, reducing the formation of bacterial biofilms, and other virulence factors [67].

Among all commercially available nanomaterials, Ag-Nps are now the most popular nanoparticles, due to their low toxicity in comparison to other nanoparticles. The initial stage of Ag-Nps nanoparticles' cytotoxic action is usually their adhesion and penetration onto microbial membrane surfaces [15]. Numerous harmful pathogens have been demonstrated to be susceptible to the antibacterial effects of silver [68]. Since ancient times, silver (Ag) has been utilized as a medicinal element; however, modern medical research is examining the properties and uses of silver nanoparticles (Ag-Nps). Silver is used in modern antibacterial salve and unguents to prevent microbiological spread of burns and open wounds. Additionally, silver is frequently utilized in implants and medical equipment composed of polymers doped with silver, a lot of silver-based consumer goods, like colloidal silver gels and fabrics with silver embedded in them, are currently utilized in athletic wear [69]. Together with various polysaccharides and essential oils, these substances greatly enhance the plant's antimicrobial qualities.

In this investigation, all isolates formed biofilms, with 53.3% exhibiting strong biofilm formation, 36.7% exhibiting moderate biofilm formation, and 10% exhibiting weak biofilm development. This study's findings were consistent with those of Freeman *et al*. [31], which is one of the primary factors that slows the healing process of burn infections. In burn infections, *Ps. aeruginosa* forms biofilms at a high rate. The optical density was found to differ significantly before and after treatment with nano-curcumin. This study examines the antibacterial and anti-biofilm properties of nanocurmin against strains of *Pseudomonas aeruginosa*, one of the major infections in Iraq.

4. CONCLUSIONS

Biosynthesise of curcumin-Ag is a clean, inexpensive, and safe method, where no toxic substances were used, and thus it has no side effects. Since many pathogenic microbes have gained resistance to antibiotics, the combination of curcumin with several nanoparticles will be helpful in treating pathogenic. The anti-bacterial activity of nano-curcumin at 256 µg/ml was significantly higher against all MDR *Ps. aeruginosa* isolates disparity with control. The phytochemical composition of turmeric, and the content of its primary biologically active compounds, most notably curcumin contribute significantly to the antimicrobial properties.

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6. ETHICAL APPROVAL

This study does not involve experiments on animals or human subjects.

7. CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

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Research Article

Clinical Features and Diagnostic Strategies for Polycystic Ovary Syndrome (PCOS) in Bahawalpur, Pakistan

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Abstract: The aim is to outline the research methodology employed for diagnosing and classifying Polycystic Ovary Syndrome (PCOS) patients while offering guidance on the management and potential cure of PCOS, ultimately facilitating the ability of affected women to conceive. The methodology in this research included multiple steps: firstly, a physical examination based on clinical features; secondly, verification of the disease using biochemical tests and ultrasound. The study included 54 female patients seeking fertility assistance, utilizing physical examination, biochemical tests, and ultrasound to diagnose PCOS. The data were collected at Bahawal Victoria Hospital, Bahawalpur, Pakistan; fifty-four patients were analyzed based on clinical features, biochemical parameters, and ultrasonography. The study revealed strong correlations between PCOS and hirsutism, oligomenorrhea, family PCOS history, and acne. Unmarried women faced a higher PCOS risk. The most common features were oligomenorrhea (90.7%) and hirsutism (83.3%), followed by amenorrhea (57.4%), family history (55.6%), and acne (53.8%). All these factors showed significant associations with PCOS ($p < 0.001$ for hirsutism, oligomenorrhea, and family history, while $p = 0.002$ for acne). A positive correlation was found among patients with PCOS who had BMI, hirsutism, a family history, and acne. The research at Bahawal Victoria Hospital, Pakistan, employed diverse diagnostic tools to observe and categorize 54 patients, demonstrating early PCOS diagnosis effectiveness and manageability through medication and lifestyle changes for infertility. This study highlights the importance of integrating clinical, biochemical, and ultrasonographic features for the accurate diagnosis of PCOS. Early diagnosis and targeted management can significantly improve outcomes for affected women.

Keywords: Polycystic Ovary Syndrome, Obesity, Oligomenorrhea, LH/FSH Ratio, Family History.

1. INTRODUCTION

Polycystic Ovary Syndrome (PCOS) is a hormonal disorder that impacts women of childbearing age, giving rise to a range of symptoms such as irregular or missing menstrual cycles, excess hair growth (hirsutism), acne, infertility, and weight gain [1]. PCOS is a multifaceted condition influenced by both genetic and environmental factors. Although the precise origin remains elusive, it is theorized that a blend of elements, encompassing genetics, hormones, and lifestyle choices, contributes to its onset [2]. The diagnosis of PCOS in women involves evaluating their symptoms, physical examination findings, ultrasound scans, and blood test results. Even though PCOS has no known cure, various treatment approaches are available to assist in symptom management and enhance a woman's overall health [3]. Oligomenorrhea is characterized by women having fewer than nine menstrual periods within a year. Frequently, it is the result of PCOS, a hormonal disorder that impacts as many as 85% of women experiencing oligomenorrhea [4]. Irregular menstrual periods are a primary symptom of PCOS. Pharmaceutical interventions can be effective in reinstating regular menstrual

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cycles in women with PCOS, even if their intention is not to conceive [5]. Teenagers with irregular periods are frequently prescribed birth control pills. Nevertheless, in cases where they cannot use birth control pills or encounter side effects, alternative medications may become necessary [6].

Obesity is a health condition characterized by an excessive accumulation of body fat, typically assessed through the calculation of body mass index (BMI), which takes into account a person's weight and height. An individual with a BMI of 30 or greater is classified as obese [7]. There is a strong association between obesity and irregular or absent menstrual periods in women diagnosed with PCOS. In fact, obesity stands out as one of the most prevalent symptoms of PCOS [8]. Researchers are continuing their efforts to precisely understand the mechanisms through which obesity contributes to irregular periods in women with PCOS. However, their current hypothesis suggests that obesity might elevate insulin levels, potentially disrupting the process of ovulation [9, 10]. The diagnosis of PCOS is made based on a combination of clinical features, ultrasound findings, and blood test results [11]. There is no single universally accepted set of criteria for diagnosing PCOS. However, diagnosis typically requires the presence of at least two of the following three features: clinical features, ultrasonography, and blood tests. The most common clinical features of PCOS are Irregular menstrual cycles, Excess hair growth (hirsutism), Acne, Weight gain and Infertility [12].The ultrasound criteria for PCOS vary from study to study. However, the most commonly used criteria are the presence of 12 or more follicles in each ovary, increased ovarian volume (>10 mL) and increased ovarian area (>5.5 cm2) [13].Blood tests can be used to measure hormone levels and rule out other conditions that can mimic PCOS. The most commonly tested hormones are Luteinizing hormone (LH), Follicle-stimulating hormone (FSH), Testosterone and Sex hormone-binding globulin (SHBG) [14].

PCOS is a prevalent endocrine disorder, affecting an estimated 10-15% of women worldwide. In South Asia, including Pakistan, the prevalence is notably high, with estimates ranging from 20-30%. PCOS presents with a spectrum of symptoms, including irregular menstrual cycles, excessive hair growth (hirsutism), acne, infertility, and metabolic disturbances such as insulin resistance and obesity. The exact etiology remains unclear, but genetic, hormonal, and lifestyle factors play crucial roles.

The diagnosis of PCOS lacks a universally accepted criterion, although the Rotterdam Criteria (requiring at least two of the three criteria: hyperandrogenism, ovulatory dysfunction, and polycystic ovarian morphology) is widely used. This study aims to examine the clinical, biochemical, and ultrasonographic features of PCOS and provide insights into effective diagnostic and management strategies. Additionally, the frequency of PCOS in the Bahawalpur region has been incorporated based on available data. The ovarian morphology of patients was observed after the initial physical examination of the patients through pelvic or transvaginal ultrasound.

2. MATERIALS AND METHODS

The study was conducted in Gynecology outdoor patient department (OPD) of Bahawal Victoria Hospital (BVH) in Bahawalpur (BWP) which is located in Punjab province of Pakistan. Clinical, hormonal and U/S features that were consecutively recorded from patients referred to outdoor patient department. These examinations were performed in the early follicular phase, between Day 2 and 5 of the menstrual cycle. The parameters such as family history, pre-menstrual detail, and physical examine were recorded according to the survey report. Diagnosis of PCOS is shown in Figure 1.

2.1. Clinical Evaluation

First, a questionnaire-based interview about premenstrual details, obstetric histories, the severity of PCOS clinical symptoms, drug use history, family history of diabetes, oligomenorrhea, amenorrhea, length of the marriage and other associated diseases were conducted as part of the clinical evaluation. Second, a physical examination was performed to check the BMI, waist-to-hip ratio (WHR), blood pressure (BP), hirsutism and acne distribution. According to recommendations, trained medical professionals examined unmarried women via trans-abdominal ultrasound and married women via transvaginal ultrasound.

2.2. Biochemical Evaluation

The biochemical evaluation involved two blood

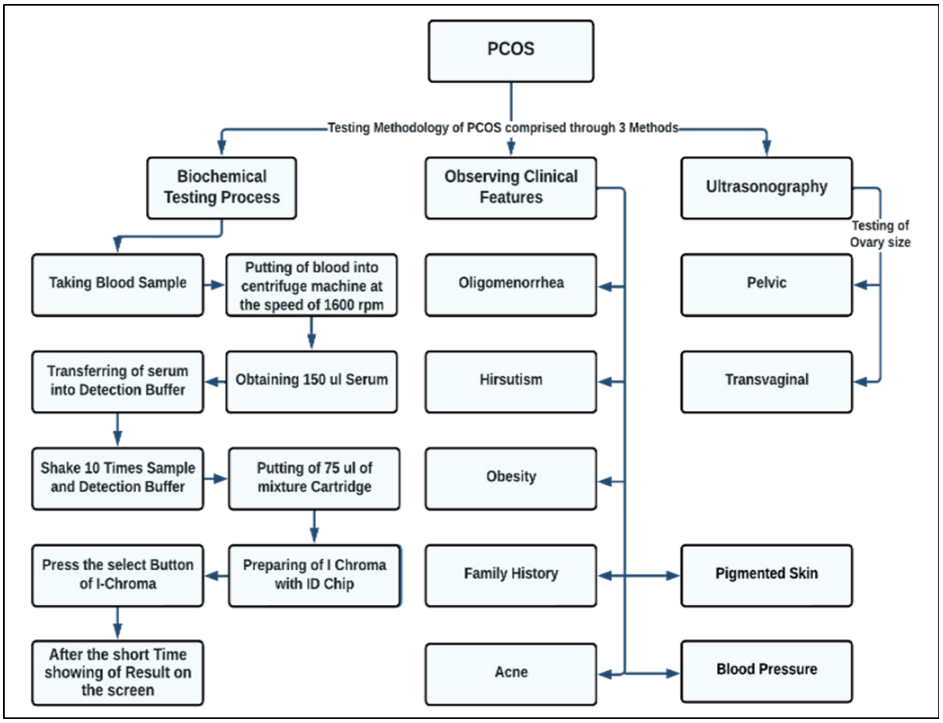

Fig. 1. Diagnosis of PCOS by Clinical Features, Biochemical Tests and Ultrasonography.

tests which were LH and FSH blood tests [15]. LH and FSH are two hormones that play a role in the menstrual cycle. LH levels are typically elevated in women with PCOS, while FSH levels are typically low.

2.3. Ultrasonography

Transvaginal Ultrasonography of ovaries was performed on five patients using a 5-9 MHz transvaginal transducer. At least one ovary with 12 follicles measuring 2 to 9 mm in size or greater than 10 mL in volume was shown on the image of the computer screen thus PCOS diagnosis was confirmed. The simplified formula for a prolate ellipsoid was used to calculate ovarian volume (OVol). OVol, follicle number (OFN) and mean follicle diameter for both ovaries were calculated and summarized. The greatest and smallest follicles' maximum sizes were measured and recorded in millimeters. Two unmarried women had her ovaries examined via transabdominal ultrasound (in this case, only OVol was assessed, without OFN).

2.4. Statistical Analysis

Descriptive statistics were utilized to depict the characteristics of PCOS in the female subjects. Frequencies, means, standard deviations were applied to investigate the relationships between BMI, LH, FSH, and LH/FSH with oligomenorrhea, acne, hirsutism, and polycystic ovaries. Data were analyzed using SPSS 25.0. Descriptive statistics were presented as means and standard deviations. Multiple logistic regression was applied to determine significant associations, adjusting for confounders such as age, BMI, and hormonal levels. Justification for using multiple logistic regression has been added. A p-value < 0.05 was considered statistically significant. Multiple logistic regression was employed to scrutinize the associations between the variables under study, with odds ratios (ORs) and their respective 95% confidence intervals (CIs) being presented. All independent variables meeting the stipulated criteria were integrated into the multiple logistic regressions. Adjustments were made for age, BMI, LH, FSH, and LH/FSH as independent variables. Statistical significance was established at a P value below 0.05.

3. RESULTS

3.1. Descriptive Analysis of Clinical and Biochemical Features in Women with PCOS

In present study, data of 54 patients fulfilled the criteria for PCOS diagnosis. All patients had physical examined and referred to their

Ultrasonography and biochemical tests. Descriptive statistics were presented for 54 polycystic ovary syndrome (PCOS) patients. Mean and standard deviation (SD) were provided for various variables. For instance, patients' marital status had a mean of 0.33, indicating around 33% were married. The average age was 27.30 ± 8.28 years, with a range of 19.02 to 35.58 years. Mean BMI was 29.04 ± 5.16, suggesting most patients were overweight or obese. Other variables like oligomenorrhea had a mean of 0.91 (91% prevalence), and hirsutism had a mean of 0.83 (83% prevalence), see Table 1.

3.2. Frequencies and Percentage of Respondents Physical Examination

The study provided distribution details for various characteristics within a group of 54 individuals. Among the individuals surveyed, 66.7% were unmarried, accounting for 36 individuals, while 33.3% were married, representing 18 individuals. Oligomenorrhea was observed in 90.7% of participants (49 individuals), whereas 9.3% (5 individuals) did not experience this condition. Hirsutism was present in 83.3% of individuals (45 cases), while 16.7% (9 individuals) did not exhibit this condition. Amenorrhea affected 40.7% of participants (22 individuals), whereas 59.3% (32 individuals) did not have this condition. Additionally, a family history of the specified condition was reported in 61.1% of cases (33 individuals), while 38.9% (21 individuals) had no family history. Acne was present in 74.1% of cases (40 individuals), whereas 25.9% (14 individuals) did not have acne. Pigmentation issues were observed in 66.7% of cases (36 individuals), while 33.3% (18 individuals) had no pigmentation issues. PCOS was reported in 51.9% of cases (28 individuals), whereas 48.1% (26 individuals) did not have PCOS.

Regarding other health issues, 57.4% of individuals (31 cases) reported no additional health concerns. Various health issues were reported, including high blood pressure, abdominal pain, infections, and other conditions. These percentages represented the breakdown of characteristics within the surveyed group of 54 individuals are given in Table 2.

Table 1. Descriptive analysis of observed women.

Clinical and biochemical features	M ± SD
Marital Status	0.33 ± 0.476
Age	27.30 ± 8.28
BMI	29.04 ± 5.16
Oligomenorrhea	0.91 ± 0.29
Hirsutism	0.83 ± 0.38
LH mlU/ml	8.11 ± 15.07
FSH mlU/ml	5.10 ± 11.22
Other issues	1.78 ± 3.14
Amenorrhea	0.41 ± 0.50
Family History	0.61 ± 0.49
Acne	0.74 ± 0.44
Headache	0.78 ± 0.42
Pigmentation	0.67 ± 0.48
PCOS	0.52 ± 0.50

3.3. Correlation of PCOS with Biochemical and Clinical Features

A t-test was conducted on 54 patients with PCOS to compare their biochemical and clinical features. The t-test results, including t-statistic, degrees of freedom, mean difference, and 95% confidence intervals, were examined. Statistically significant mean differences were observed in the following features:

Age: Patients with PCOS had a significantly higher mean age compared to those without PCOS. The mean difference was 27.286 years (95% CI: 25.03 to 29.55). BMI: Patients with PCOS had a significantly higher mean BMI than those without PCOS. The mean difference was 29.0295 (95% CI: 27.6210 to 30.4381). Oligomenorrhea: Patients with PCOS had a significantly lower mean number of menstrual cycles per year compared to those without PCOS. The mean difference was 0.897 (95% CI: 0.82 to 0.98). Normal ranges for LH and FSH in women of reproductive age are 2-10 mIU/mL and 3-20 mIU/mL, respectively. In PCOS patients, LH levels are typically elevated (> 10 mIU/mL), while FSH levels remain within the normal range.

Table 2. Frequencies and percentages of clinical features.

Clinical features	Frequency	Percent
Marital status		
Unmarried	36	66.7
Married	18	33.3
Total	54	100.0
Oligomenorrhea		
No	5	9.3
Yes	49	90.7
Total	54	100.0
Hirsutism		
No	9	16.7
Yes	45	83.3
Total	54	100.0
Amenorrhea		
No	32	59.3
Yes	22	40.7
Total	54	100.0
Family history		
No	21	38.9
Yes	33	61.1
Total	54	100.0
Acne		
No	14	25.9
Yes	40	74.1
Total	54	100.0
Pigmentation		
No	18	33.3
Yes	36	66.7
Total	54	100.0
PCOS		
No	26	48.1
Yes	28	51.9
Total	54	100.0
Other health issues		
No	31	57.4
BP High	8	14.8
Lower abdomen pain	4	7.4
Secondary Amenorrhea	1	1.9
Uterus size enlarged	1	1.9
Vaginal discharge	2	3.7
Heavy Bleeding (Hb=7)	1	1.9
UTI	1	1.9
Intestinal infection	1	1.9
Renal infection	1	1.9
Diabetes	1	1.9
Hypotheroidism	1	1.9
Vaginal infection	1	1.9
Total	54	100.0

LH mlU/ml: Patients with PCOS had a significantly higher mean LH level than those without PCOS. The mean difference was 8.096 (95% CI: 3.983 to 12.210). FSH mlU/ml: Patients with PCOS had a significantly higher mean FSH level than those without PCOS. The mean difference was 5.090 (95% CI: 2.026 to 8.153). Hirsutism: Patients with PCOS had a significantly higher mean hirsutism score than those without PCOS. The mean difference was 0.823 (95% CI: 0.72 to 0.93), see Table 3.

The study focused on women aged 18-40 years diagnosed with Polycystic Ovary Syndrome (PCOS) based on the Rotterdam Criteria, with exclusion criteria applied to patients with other endocrine disorders, chronic illnesses, or recent hormonal therapy. Clinical assessments included detailed history-taking and physical examinations, covering BMI, blood pressure, hirsutism assessment using the Ferriman-Gallwey Score, acne severity, and menstrual history. Biochemical evaluations were conducted on fasting blood samples, analyzing Luteinizing Hormone (LH), Follicle-Stimulating Hormone (FSH) levels to assess insulin resistance. Ultrasonographic assessments were performed using transvaginal ultrasound for married women and transabdominal ultrasound for unmarried women to evaluate ovarian morphology. Polycystic ovaries were identified by an ovarian volume greater than 10 mL, the presence of 12 or more follicles measuring 2-9 mm in each ovary, and a detailed ultrasound protocol was followed to ensure accuracy.

3.4. Pearson Correlation Coefficient between Biochemical and Clinical Features

A Pearson correlation coefficient was used to analyze the relationships between various biochemical and clinical features in 54 patients with polycystic ovary syndrome (PCOS). Notable correlations included: A positive correlation between age and BMI suggested that older patients tended to have higher BMIs, possibly due to increased likelihood of being overweight or obese. A negative correlation between marital status and amenorrhea, indicating unmarried patients were more prone to irregular menstrual cycles. A positive correlation between hirsutism and amenorrhea, suggesting patients with more hirsutism also experienced amenorrhea due to shared androgen excess symptoms. A positive

Table 3. T-Test of biochemical and clinical features.

Biochemical and clinical features	t	df	Mean difference	Confidence interval	
				Lower	Upper
Age	24.204	53	27.286	25.03	29.55
BMI	41.337	53	29.0295	27.6210	30.4381
Oligomenorrhea	22.539	53	0.897	0.82	0.98
LH mlU/ml	3.947	53	8.096	3.983	12.210
FSH mlU/ml	3.332	53	5.090	2.026	8.153
Hirsutism	16.083	53	0.823	0.72	0.93
Marital Status	4.993	53	0.323	0.19	0.45
Amenorrhea	5.888	53	0.397	0.26	0.53
Family history	8.977	53	0.601	0.47	0.74
Acne	12.140	53	0.731	0.61	0.85
Headache	13.445	53	0.768	0.65	0.88
Pigmentation	10.141	53	0.657	0.53	0.79
PCOS	7.409	53	0.509	0.37	0.65

* 95% Confidence interval of the difference.

correlation between family history and PCOS suggested a genetic link between PCOS and family history. Positive correlation between acne and PCOS, as acne and androgen excess symptoms were related, and both can indicate PCOS (Table 4).

3.5. Presentation of ultrasonography

PCOS was diagnosed through pelvic and trans-vaginal ultrasound. Seven patients had greater ovarian volume (> 10ml) in the right ovary, left ovary, or both ovaries mentioned as yes or No. Many follicles were counted during the ultrasonography in both ovaries, or one had 10-12 or > 12 number of follicles. 4 patients had greater than 12 follicles and remaining had less than 12 (Table 5). Out of 7 patients, 5 had right ovary volume > 10ml, 3 had left ovary volume > 10ml and 1 patient had normal ovary size.

3.6. Patient Characteristics

Among the 54 PCOS patients, 33.3% were married, and 66.7% were unmarried. The mean BMI was 29.04 ± 5.16, indicating a high prevalence of overweight and obesity. Oligomenorrhea (90.7%) and hirsutism (83.3%) were the most common clinical features, followed by acne (74.1%) and amenorrhea (40.7%). A strong familial predisposition was noted, with 61.1% reporting a positive family history of PCOS.

3.6.1. Biochemical and ultrasonographic findings

The LH/FSH ratio was elevated in 70.6% of cases (mean LH = 8.11 IU/mL, mean FSH = 5.10 IU/mL). Hyperandrogenism was confirmed in 68.5% of patients via elevated testosterone levels. In ultrasound findings, 75% of patients had polycystic ovarian morphology, with an average ovarian volume exceeding 10mL. Grouping of patients and control details have been clarified.

3.6.2. Correlations and risk factors

Higher BMI was found to be correlated with increased fasting insulin and glucose levels ($p < 0.01$), supporting the link between obesity and

Table 4. Correlation between clinical and biochemical features.

		BMI	LH mlU/ml	FSH mlU/ml
Age	Pearson Correlation	0.213	0.067	0.367**
	Sig. (2-tailed)	0.122	0.632	0.006
Marital status	Pearson Correlation	-0.213	-0.154	-0.281*
	Sig. (2-tailed)	0.123	0.266	0.040
Oligomenorrhea	Pearson Correlation	-0.073	-0.085	0.043
	Sig. (2-tailed)	0.599	0.539	0.757
Hirsutism	Pearson Correlation	-0.027	0.004	0.010
	Sig. (2-tailed)	0.846	0.978	0.943
Amenorrhea	Pearson Correlation	-0.147	-0.450**	-0.380**
	Sig. (2-tailed)	0.287	0.001	0.005
Family history	Pearson Correlation	0.039	0.329*	.297*
	Sig. (2-tailed)	0.780	0.015	0.029
Acne	Pearson Correlation	-0.086	-0.087	-0.133
	Sig. (2-tailed)	0.535	0.532	0.336
Pigmentation	Pearson Correlation	0.153	0.004	-0.250
	Sig. (2-tailed)	0.270	0.974	0.069
PCOS	Pearson Correlation	0.156	0.345*	0.296*
	Sig. (2-tailed)	0.259	0.011	0.030
Headache	Pearson Correlation	0.041	-0.140	-0.181
	Sig. (2-tailed)	0.767	0.313	0.189
Other issues	Pearson Correlation	0.029	-0.023	-0.009
	Sig. (2-tailed)	0.835	0.866	0.946

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

Table 5. Polycystic ovaries on ultrasonography.

No. of patients (n = 7)	Right ovary > 10ml	Left ovary > 10ml	Normal ovary	No. of follicles
1	yes	No	No	>14
2	yes	Yes	No	Multiple
3	No	No	Yes	8-10
4	yes	No	No	14-15
5	yes	No	No	Multiple
6	No	Yes	No	>12
7	yes	Yes	No	14-16

metabolic disturbances in PCOS. Unmarried women exhibited higher PCOS prevalence, potentially due to delayed diagnosis and cultural factors.

4. DISCUSSION

Irregular menstrual cycles and a range of symptoms, along with significant changes in the LH/FSH ratio, are attributed to polycystic ovary syndrome (PCOS) [16]. Managing PCOS involves targeting endocrine and biochemical factors, which encompasses addressing concerns such as hyperinsulinemia, insulin resistance, and the LH/FSH ratio, as supported by the findings in this study. In our findings, 68.51% of patients were obese, which contrasts with the 50.4% [17] in the study. Notably,

the women in our study had a comparatively younger mean age. Disparities in the levels of LH and FSH production, with a higher LH/FSH ratio emerging as the predominant clinical feature among women diagnosed with PCOS [18]. A heightened LH/FSH ratio was observed in 70.58% of women diagnosed with PCOS. Consequently, the authors propose that the LH/FSH ratio represents one of the distinctive features of women with PCOS [19]. However, no correlation was detected between LH and FSH levels in conjunction with the LH/FSH ratio in our initial observations [20]. However, our study documented a substantial link between hormone levels and the LH/FSH ratio. This connection may be attributed to the LH levels, potentially explaining the positive association with LH and the negative association with FSH.

A substantial portion of participants as having polycystic ovary syndrome (PCOS). Among these individuals, 31.4% displayed acne, and 78.9% exhibited hirsutism [20]. These prevalence rates exceeded those documented in earlier research. Furthermore, individuals with PCOS who were undergoing treatment exhibited a higher incidence of irregular menstrual cycles, acne, and hirsutism. The authors posit that a study involving a larger sample size and extended longitudinal monitoring would yield more conclusive findings. Within our study, individuals diagnosed with PCOS underwent treatment involving metformin, resulting in reduced glucose levels and enhanced insulin sensitivity. Additionally, letrozole and clomiphene were employed to induce ovulation in infertile women. While health enhancements were observed throughout the treatment, a disparity in the prevalence of acne and hirsutism was discernible between those who received treatment and those who did not. Treatment of patients with metformin and inositol leads to decreased glucose levels and increased insulin sensitivity [21].

There were 93% of daughters whose mothers had PCOS also experienced PCOS themselves [22]. Additionally, PCOS and about 50% of the sisters of women affected by PCOS exhibited hyperandrogenemia [23]. Through the application of linear regression, it was determined that among patients receiving PCOS treatment, the consistency of menstrual cycles was notably influenced by the LH/FSH ratio. Women with regular menstrual cycles had a lower LH/FSH ratio compared to those with irregular cycles. In our current investigation, it was identified a prevalence rate of 61% among females with PCOS who had both mothers and sisters with the condition.

5. CONCLUSIONS

PCOS is a multifaceted condition requiring early diagnosis and tailored treatment. This study highlights the importance of incorporating clinical, biochemical, and ultrasonographic markers for an accurate diagnosis. Managing obesity and insulin resistance should be prioritized to improve patient outcomes. Further research with larger sample sizes is needed to validate these findings.

6. ETHICAL STATEMENT

This study was approved by the Ethical Review Committee of The Islamia University of Bahawalpur, Pakistan. Written informed consent was obtained from all participants before their inclusion in the study.

7. ACKNOWLEDGEMENTS

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8. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Effect of Plant Geometry on the Growth and Yield of Sugar Beet (*Beta vulgaris* L) cv. California-KWS: A Study on Inter-row and Intra-row Spacing

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Abstract: This study was aimed to assess the effect of plant geometry on the growth and yield of sugar beet under the agroclimatic conditions of D. I. Khan, Pakistan. Sugar beet, hybrid cv. California-KWS from Al-Moiz sugar mills (Dera Ismail Khan) was planted two seeds per hole on ridges at an inter-and intra-row spacing: 20 × 50, 20 × 60, 30 × 50, 30 × 60, 40 × 50, 40 × 60, 50 × 50 and 50 × 60 cm. The experiment was carried out in a Randomized Complete Block Design and replicated thrice. The parameters like plant height (cm), number of leaves, leaf area plant^{-1} (cm^2), leaf and root weights per plant, root length and diameter (cm), TSS% (total soluble solids), sucrose (%), root and sugar yields (t ha^{-1}) were studied. Results revealed that significantly maximum plant height, leaf area, leaf and root weights were observed in 40 × 50 cm spacing, number of leaves were maximum in 30 × 50 cm spacing, maximum root diameter was observed in 50 × 50 cm spacing while maximum root length, sucrose content, TSS, root and sugar yield were observed in 20 × 50 cm. Hence, the results showed that 20 × 50 cm is best spacing for obtaining highest yield and quality of sugar beet crop in the area.

Keywords: Sugar Beet, Planting Geometry, Quality, Root Yield, Sugar Yield.

1. INTRODUCTION

Sugar beet (*Beta vulgaris* L.) being an important crop, after sugar cane, produces 30% sugar annually worldwide. Europe contributes more than 60% of the sugar beet production area [1]. In Pakistan, sugar cane is the major source of sugar production while sugar beet share is small. The beet plant contains crown, neck, and root [2]. 16-20% sucrose is present in the roots [3]. Its leaves also contain carbohydrates, protein, vitamin A and green manure [4]. Sugar beet can be cultivated on loam and clay loam soils and it can tolerate alkaline conditions and also resist cold and drought [5]. During 2022-23, the area under sugar beet cultivation was 6426 hectares, producing 480087 tons beets in Pakistan, while in Khyber Pakhtunkhwa, area under its cultivation was 821 hectares with annual production of 32454 tons [6]. In Pakistan, it is cultivated in Peshawar, Charsadda, Mardan and D.I. Khan (Khyber Pakhtunkhwa). However, it has also been cultivated in Punjab, Sindh and parts of Balochistan. Due to decrease in water availability, the area under cultivation of sugar cane is decreasing in Pakistan, therefore, sugar beet is a suitable solution under such circumstances, having potential of giving two-time more sugar yield and more financial returns as compared to sugar cane in a short (5-6 months) period [7]. Factors like environmental conditions, soil, cultural practices, weeds, pests and diseases

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etc. are critical for sugar beet cultivation. Plant density or plant population is also one of the most crucial factors. Reduced germination, time of sowing, poor field preparation and several biotic and abiotic factors are the main causes which can reduce plant numbers per unit area [8, 9]. Sucrose contents, purity and sugar yield were significantly increased by the increase in plant population from 87,500 to 100,000 plants ha^{-1}[10]. El-Sarag [11], Bhullar *et al*. [12], Shukla and Awasthi [13] and Sadre *et al*. [14] reported maximum yield of both roots and sugar at higher plant density.

Around 90,000 to 110,000 plants ha^{-1} are recommended for sugar crop [15-18]. New varieties have straight leaves that promote growth even in smaller area, so there is a possibility of even higher population. To find out the best plant density for proper development of higher mass and quality under different field conditions is crucial [19]. In Pakistan, agronomic studies especially of plant populations in sugar beet are rare. In Pakistan there are a few studies [20, 21] about sugar beet density, therefore, it is dire need of the day to asses proper plant density of this most important crop of the area. Hence, a study was designed with the objective to find a suitable plant density for such an important crop of the area.

2. MATERIALS AND METHODS

2.1. Study Site

The trial was performed in Faculty of Agriculture, Gomal University D. I. Khan during winter season (2013-14 and 2014-15). The soil properties and the weather data of the research site was same as reported previously [22].

2.2. Design and Treatments

Sugar beet cultivar California-KWS was planted two seeds per hole on ridges, in scheme of RCBD with three replications. Sowing was performed on October 16, 2013 and on October 17, 2014 at an inter-and intra-row spacing: 20 × 50, 20 × 60, 30 × 50, 30 × 60, 40 × 50, 40 × 60, 50 × 50 and 50 × 60 cm. Triple super phosphate and potassium sulphate were supplied at a basal rate of 100 and 62.5 kg ha^{-1}, respectively; while N was applied in two split doses (30 days after sowing and 60 days after sowing) at 120 kg ha^{-1}. Field was immediately irrigated after seeding and then at an interval of fortnight. All field cultural practices were conducted as per requirement.

2.3. Parameters Observed

The data of the following parameters were observed as:

2.3.1. Plant height (cm) was measured with measuring tape by taking ten plants at random from each replication and mean was calculated.

2.3.2. Number of leaves per plant: Mean of randomly counted leaves of ten (10) plants was calculated from each replication.

2.3.3. Leaf area per plant (cm^2): Area of the leaves was assessed as per Ahmad *et al*. [21].

2.3.4. Leaf and root weight per plant were determined by a digital scale.

***2.3.5. Root length (cm)*:** Measuring tape was used to record the length.

2.3.6. Root diameter (cm) was calculated as stated by Ahmad *et al*. [21].

2.3.7. Total soluble solids (%) were measured as per Horwitz and Latimer [23].

2.3.8. Sucrose (%) was determined by Lane and Eynon method as per Horwitz and Latimer [23].

2.3.9. Root and sugar yields (t ha^{-1}) were assessed as per Khan *et al*. [22].

The common cultural practices for growing sugar beet were followed accordingly. Sugar beet crop was harvested at its maturity. Roots were dugout manually by using a commonly used agricultural tool “Khurpa”.

2.4. Statistical Analysis

The data was subjected to ANOVA as stated by Steel *et al.* [24] using Statistics 8.1 software. The values were compared by using LSD test at $P \leq 0.05$ levels.

3. RESULTS AND DISCUSSION

3.1. Plant Height (cm)

The plant geometry significantly affected the plant height (cm) during both years (Table 1). The tallest plants (44.68 and 44.66 cm) were recorded in 40 × 50 cm while shortest plants (42.44 and 42.45 cm) were observed in 50 × 60 cm during both years. By increaing space up to a certain limit might improve vegetative growth. Khogali *et al*. [25], Bacha *et al*. [26], Maboko and Du Plooy [27], Imran *et al.* [28], Sharifi and Namwar [29] and Wu *et al.* [30] also reported similar results in various crops.

3.2. Number of Leaves per Plant

The leaves count was significantly affected by plant geometry during both years (Table 1). Higher leaf count plant^{-1} (45.57 and 45. 60) was recorded in 30 × 50 cm while minimum (42.67 and 42.50) was recorded in 50 × 60 cm during both years. By increasing space up to a certain limit might improve top/leaves growth. Results are in line with Khogali *et al.* [25], Imran *et al.* [28] and Zahoor *et al.* [31].

3.3. Leaf Area per Plant (cm^2)

Leaf area was significantly affected by the plant geometry during both years (Table 1). Among all treatments, the maximum leaf area (445.24 and 444.94 cm^2) was recorded in 40 × 50 cm and minimum leaf area (435.41 and 434.60 cm^2) was recorded in 50 × 60 cm during both years. The results might be due to better light use efficiency by plants which resulted in superior vegetative growth (plant height, leaf length). Results agree with those obtained by Soleymani and Shahrajabian [16], Wu *et al.* [30], Zahoor *et al.* [31] and Varga *et al.* [32].

3.4. Leaf Weight (g) per Plant

Leaf weight was significantly affected by the plant geometry during both years (Table 1). Maximum leaf weight (479.66 and 480.20 g) was found in 40 × 50 cm while minimum (478.40 and 477.54 g) was recorded in 50 × 60 cm during both years. It could be due to better vegetative growth and better available resources for plants. The findings strongly agree with Varga *et al.* [33].

Table 1. Effect of plant geometry on plant height (cm), number of leaves, leaf area (cm^2) and leaf weight per plant (g).

Year I				
Plant density	**Plant height (cm)**	**Number of leaves**	**Leaf area (cm^2)**	**Leaf weight (g)**
20 × 50	43.72 c	43.57 c	442.45 c	478.79 d
20 × 60	43.69 d	43.53 c	441.79 d	478.69 e
30 × 50	44.41 b	45.57 a	443.47 b	479.46 b
30 × 60	44.43 b	45.50 a	438.81 e	479.29 c
40 × 50	44.68 a	44.43 b	445.24 a	479.66 a
40 × 60	44.66 a	44.53 b	442.44 c	479.46 b
50 × 50	42.44 e	42.73 d	437.45 f	478.48 f
50 × 60	42.44 e	42.67 d	435.41 g	478.40 g
LSD	0.026	0.125	0.100	0.051
Year II				
20 × 50	43.70 e	43.63 c	442.45 c	478.82 d
20 × 60	43.57 f	43.50 c	441.76 d	478.70 e
30 × 50	44.40 c	45.60 a	443.47 b	479.46 b
30 × 60	44.34 d	45.56 a	438.81 e	479.29 c
40 × 50	44.66 a	44.50 b	444.94 a	480.20 a
40 × 60	44.59 b	44.60 b	442.44 c	479.46 b
50 × 50	42.46 g	42.77 d	436.60 f	478.48 f
50 × 60	42.45 g	42.50 e	434.60 g	477.54 g
LSD	0.036	0.146	0.115	0.057

Response of three replicates. Means bearing different letters in the same column (years) are significant ($P \leq 0.05$).

3.5. Root Weight (g) per Plant

Significantly heaviest roots (1331.8 and 1361.8 g) were recorded in 40 × 50 cm while minimum root weight (1148.7 and 1148.1 g) was recorded in 50 × 60 cm during both years (Table 2). It could be due to better root growth and better available resources for plants. The findings agree with Ahmad *et al.* [21], Varga *et al.* [33] and Nafei *et al.* [34].

3.6. Root Length (cm)

The root length was significantly affected by plant geometry during both years (Table 2). The lengthiest roots (36.76 and 36.80 cm) were recorded in 20 × 50 cm while the shortest roots (34.39 and 34.27 cm) were observed in 50 × 60 cm during both years. By decreasing space, roots might have grown vertically to have maximum root length (compared to wider spacing). The results are in agreement with Nafei *et al.* [34] and Hozayn *et al.* [35].

3.7. Root Diameter (cm)

The root diameter (cm) was positively affected by plant geometry during both years (Table 2). Thickest roots (12.62 and 12.64 cm) were observed in 50 × 50 cm while thinnest roots (11.51 and 11.55 cm) were recorded in 20 × 50 cm during both years. It could be due to better utilization of soil and other resources due to less plant density. The results are in accord with Sadre *et al.* [14], Varga *et al.* [33], Hozayn *et al.* [35] and Leilah *et al.* [36].

3.8. Sucrose Content

The plant geometry significantly affected the sucrose content during both years (Table 2). Higher sucrose content (16.40 and 16.45%) was recorded in 20 × 50 cm while minimum (15.96 and 15.87%) was observed in 50 × 60 cm during both years. Findings are in line with Ahmad *et al.* [21], Varga *et al.* [32], Nafei *et al.* [34] and Hozayn *et al.* [35].

3.9. Total Soluble Solids (TSS%)

TSS% was significantly affected by plant geometry during both years (Table 3). Maximum TSS (19.14 and 19.17%) were recorded in 20 × 50 cm while minimum (18.70 and 18.74%) was recorded in

Table 2. Effect of plant geometry on root weight (g), root length (cm), root diameter (cm) and sucrose%.

Year I				
Plant density	**Root weight (g)**	**Root length (cm)**	**Root diameter (cm)**	**Sucrose%**
20 × 50	1173.6 d	36.76 a	11.51 f	16.40 a
20 × 60	1160.8 e	36.73 ab	11.61 e	16.38 a
30 × 50	1230.9 b	36.66 bc	11.72 d	16.30 b
30 × 60	1213.0 c	36.59 c	11.72 d	16.26 c
40 × 50	1331.8 a	35.56 d	11.94 b	16.22 d
40 × 60	1229.8 b	35.43 e	11.82 c	16.14 e
50 × 50	1149.9 f	34.56 f	12.62 a	16.02 f
50 × 60	1148.7 f	34.39 g	12.60 a	15.96 g
LSD	9.211	0.102	0.076	0.023
Year II				
20 × 50	1173.6 d	36.80 a	11.55 f	16.45 a
20 × 60	1156.2 e	36.75 ab	11.62 e	16.34 b
30 × 50	1230.9 b	36.69 bc	11.72 d	16.30 c
30 × 60	1215.6 c	36.59 c	11.72 d	16.26 d
40 × 50	1361.8 a	35.56 d	11.94 b	16.22 e
40 × 60	1232.5 b	35.43 e	11.84 c	16.14 f
50 × 50	1149.1 e	34.56 f	12.64 a	16.05 g
50 × 60	1148.1 e	34.27 g	12.58 a	15.87 h
LSD	8.741	0.102	0.054	0.033

Response of three replicates. Means bearing different letters in the same column (years) are significant ($P \leq 0.05$).

50 × 60 cm during both years. It might be due to development of poor-quality plants due to decreased population and increased non-sugar contents [36]. The findings agree with Ahmad *et al.* [21], Nafei *et al.* [34] and Hozayn *et al.* [35]. Wu *et al.* [30] reported similar results in Perilla.

3.10. Root Yield (t ha^{-1})

The plant geometry significantly affected root yield (t ha^{-1}) during both years (Table 3). Maximum root yield (63.43 and 63.46 t ha^{-1}) was recorded in 20 × 50 cm whereas, minimum root yield (60.26 and 60.66 t ha^{-1}) was found in 50 × 60 cm during both years. It could be due to high light interception which contributed positively to photosynthesis with relative increase in root numbers per hectare. The findings agree with Bhullar *et al.* [12], Sadre *et al.* [14], Soleymani and Shahrajabian [16], Varga *et al.* [32], Nafei *et al.* [34], Hozayn *et al.* [35], Leilah *et al*. [36] and Xu *et al*. [37].

3.11. Sugar Yield (t ha^{-1})

Sugar yield was also significantly affected by the plant geometry during both years (Table 3). Maximum sugar yield (10.40 and 10.47 t ha^{-1}) was noted in 20 × 50 cm while minimum (9.62 and 9.70 t ha^{-1}) was recorded in 50 × 60 cm during both years. The sugar yield relates with root yield (rather than change of technological quality of roots). Results agree with Masri [10], Bhullar *et al.* [12], Sadre *et al.* [14], Ahmad *et al.* [21], Nafei *et al.* [34], Hozayn *et al*. [35] and Xu *et al*. [37].

4. CONCLUSIONS

Results showed that plant geometry significantly affected almost all the studied characters of sugar beet during the study. In general, the sugar beet plants having higher crop densities produced a higher yield and quality as compared to lower densities. The lengthiest roots, highest sucrose, TSS, root and sugar yield were observed in 20 × 50 cm spacing. Hence, it is concluded that sowing of sugar beet at 20 × 50 cm spacing is recommended for better growth, yield and quality of the crop in the area.

Table 3. Effect of plant geometry on TSS (%), root and sugar yield (t ha^{-1}).

Year I			
Plant density	**TSS%**	**Root yield (t ha^{-1})**	**Sugar yield (t ha^{-1})**
20 × 50	19.14 a	63.43 a	10.40 a
20 × 60	19.12 a	63.35 a	10.38 b
30 × 50	19.05 b	62.72 b	10.22 c
30 × 60	19.00 c	62.70 b	10.16 d
40 × 50	18.95 d	61.76 c	10.02 e
40 × 60	18.87 e	61.46 d	9.92 f
50 × 50	18.73 f	60.28 e	9.66 g
50 × 60	18.70 f	60.26 e	9.62 h
LSD	0.030	0.100	0.020
Year II			
20 × 50	19.17 a	63.46 a	10.47 a
20 × 60	19.08 b	63.40 a	10.36 b
30 × 50	19.05 c	62.72 b	10.23 c
30 × 60	18.97 d	62.70 b	10.19 d
40 × 50	18.95 d	61.66 c	9.99 e
40 × 60	18.87 e	61.70 c	9.96 f
50 × 50	18.77 f	60.69 d	9.74 g
50 × 60	18.74 f	60.66 d	9.70 h
LSD	0.026	0.117	0.026

Response of three replicates. Means bearing different letters in the same column (years) are significant ($P \leq 0.05$).

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6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

A Quality Assessment of Commonly Vended Tomato, Plum and Mint Sauces in Kamoke, Pakistan

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Abstract: Sauces are food additives that are frequently consumed globally and especially with fast food in Pakistan but often their consumer quality related issues are noticed. That is why; current study was designed to examin selective physicochemical properties and microbial contamination in commonly vended tomato, plum and mint sauces in Tehsil Kamoke, District Gujranwala, Punjab, Pakistan. In this experimentation, total 15 samples were collected (5 samples of each plum, mint and tomato sauces) from a uniform distance of 2 km accorAding to the map of Kamoke. For microbial culture based analysis, selective tests were done to mainly detect coliforms and results were statistically evaluated by applying one way ANOVA. The physicochemical parameters like pH and sugar content were found significant at 0.01% level for sauces of mint (4.32%) and for plum (15.54%), respectively. Among artificial sweeteners aspartame was totally absent in all samples but saccharine was present in some samples in greater amount than standard consumption values. The mint sauce samples were found contaminated (488CFU/ml) at 5% significance level. Similarly, for coliforms (472 to 568.6 CFU/ml) of selected locally vended plum, mint and tomato sauces were noted. Microbial contaminants included *Staphylococcus epidermis*, *Bacillus simplex, Escherichia coli, Salmonella spp, Arthrobacter dextranolyticus, Pseudomonas cidrella, Bacillus weihenstephanesis, Enterobacter aerogenus, Klebsiella pneumonia, Pseudomonas aeroginosa* and *Proteus mirabilis*. During sampling, it was observed that mostly sauces were exposed to high temperature and were placed uncovered. Vendors were unaware of personal hygiene, proper handling, storage and quality control standards for sauces which results in adulteration and contamination. It is recommended that to improve the quality of commonly vended sauces, their regular inspection along with the programmed general public awareness should be organized.

Keywords: Sauces, Quality Control, Adulteration, Contamination, Public Awareness.

1. INTRODUCTION

Sauces are gravies which serve as appetizers and as flavor enhancing agents so they are commonly served along with fried items and other cuisines in every region of the world. However, in poorly developed counties, these sauces are usually not prepared and served by following rules of food authority and general public hygiene. Though they are freshly prepared but usually served without addition of food preservatives, so have poor shelf life and they are sold for unlimited duration with improper storage which may result in foodborne diseases in consumers due to their intake [1].

Similarly, in currently selected region of Punjab Pakistan, these locally prepared and vended sauces are always in high demand to their good taste and economical price. But reported data indicated that almost half of the local venders of sauces are located near to sewerage drains and garbage dumps, in much crowded places and such surrounding environment greatly promotes the microbial growth and foodborne pathogenesis [2]. The way tomato sauce is quite popular one around the globe but due to poor hygienic practice and handling by local venders, often found loaded with microbial contamination, e.g., *E. coli, Listeria spp, Salmonella spp, Shigella spp, Vibrio spp,*

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Staphylococcus spp, Streptococcus spp. along with the presence of other adulterants like paprika seeds, corn starch, sucrose and salt [3].

Moreover, these plum, tomato and mint sauces are often prepared by using dusty and poor quality ingredients like chili, onion, red and green tomatoes and coriander which may contain enteric pathogens causing disease outbreaks and health complications like diarrhea and malnutrition [4, 5]. In this regard, most commonly reported Gram negative bacterial genera include *Escherichia, Vibrio, Shigella, Salmonella, Kelbsiella* and a supportive factor is availability of higher temperature in surrounding environment which may not only alter the biochemical composition of sauces but also directly promotes the microbial growth [6]. Therefore, the current study was conducted to check quality of locally vended sauces of high demand in selected region because they may inflence the health of consumers, if not prepared and severed according to quality control standereds.

2. MATERIALS AND METHODS

2.1. Study Area

This study was conducted in the Tehsil Kamoke (District Gujranwala, Punjab, Pakistan). The local food venders along road sides are found quite common in selected region, that is why; total 15 samples (5 samples of each plum, mint and tomato sauces) were collected from 5 random locations of Tehsil Kamoke at a uniform distance of 2 km according to the map of this region. These samples were assigned with codes: Plum sauce samples (A, B,C, D, E), mint sauce samples (F, G, H, I, J) and tomato sauce samples (K, L M, N, O).

2.2. Study Design

This experimental study was conducted for the evaluation of physicochemical properties and microbial contamination in commonly vended tomato, plum and mint sauces of Tehsil Kamoke.

2.3. Physicochemical Analysis

2.3.1. Ash content determination by microwave oven method

For this, in 1 g of each sauce sample 2 ml of H_2O_2 was added and this mixture was microwaved at each power (low, high and medium) up to 3 minutes to dry. Later on, allowed to cool down and then 1.5 ml of distilled water was added into dried sample and mixed it well. This prepared sample was added into labeled ependroff and it was centrifuged at 5000 rmp for one hour then the weight of obtained pallet was calculated [7] by following Equation (1):

$$\%\ of\ ash\ content = \frac{W3-W1}{W2-W1} \times 100 \quad (1)$$

2.3.2. Evaluation of pH and electrical conductivity (EC)

The pH and electrical conductivity of all samples were determined in triplicate manner with digital pH and EC meter [8, 9].

2.3.3. Evaluation of acidity

To determine acidity by titration method, 10ml of each sauce sample were titrated against NaOH (0.1 N) solution after the addition of 2-3 drops of phenolphthalein indicator until the color of solution was turned into purple and calculations were done [11] by using Equation (2):

$$Acidity = \frac{Amount\ of\ NaOH \times Conc.\ of\ NaOH \times 1.5}{Amount\ of\ sample} \quad (2)$$

2.3.4. Detection of artificial sweeteners

2.3.4.1. Brix test:

A thick fine layer of each sample was placed on the refractometer and reading of percentage sugar was noted from scale. On the basis of results of this test, saccharine test was further performed only for those samples for which < 5% reading was noted which were an indication that artificial sweeteners were might be present [12].

2.3.4.2. Saccharine test:

This test was performed only for those sauces which have < 5% sugar content and for this purpose, 1g sauce sample was dissolved 100 ml distilled water and placed for 1hr. shaking. After that the prepared solution was centrifuged at 8400 rmp speed for 10 minutes at 20 °C. The obtained supernatant was used to measure optical density by spectrophotometer and further calculations were done [12] by employing Equation (3):

$$Percentage = \frac{\%\ of\ Saccharine \times 100}{Specific\ absorbance \times wt} \quad (3)$$

2.3.4.3. Aspartame test:

To perform aspartame test, 20 g of each sample were dried in oven then 20 ml chloroform and 5 ml acetate buffer were added into each sample and placed in shaker for 45 minutes. After that filtration of each prepared solution was done and obtained filtrates were dried on hot plate. After drying, 5 ml ninhydrin solution was added into each sample containing flask and again dried them on hot plate. No color was appeared in all which indicated that samples had no aspartame content [12].

2.4. Detection of Microbial Contamination

For the detection of microbial contamination, following tests were performed:

2.4.1. By using nutrient agar

Nutrient agar plates were prepared in triplicates for all 15 samples and spreading of 1ml of each sauce sample was done and plates were incubated at 37 °C for 48 hrs. After that plates were examined for probable bacterial colonies identification by observing morphological features and colony forming units (CFU/ml) were also determined [13]. For microbial colonies probable identification and confirmation, following biochemical tests were performed:

2.4.1.1. Catalase test:

On a clean glass slide, small amount of sample from each obtained microbial colony on nutrient agar was placed then 2 drops of H_2O_2 were poured on it and changes were noted [14].

2.4.1.2. Gram staining:

Separate microbial smear was prepared for each detected strain. It was air dried and heat fixed then its staining was done according to the manual [15].

2.4.1.3. Amylase test:

Each detected microbial contaminant was isolated on separate nutrient agar plate and 2 to 3 drops of 10% iodine solution were added on it and waited for 10 minutes to note observations and the results were noted that either microbe is amylase producer or not [16].

2.4.2. Coliforms detection by using EMB agar

To detect the presence of fecal microbial contamination, eosin methylene blue (EMB) agar plates were prepared in triplicates for all 15 samples and spreading of 1ml of each sauce sample was done and plates were incubated at 37 °C for 48 hrs. After that plates were examined for probable bacterial colonies identification by observing morphological features and colony forming units (CFU/ml) were also determined [17].

3. RESULTS AND DISCUSSION

3.1. Physicochemical Analysis

3.1.1. Ash content

The sample K of mint sauce had the lowest ash contents whereas J and M samples of plum sauce and in case of tomato sauce, sample O had highest ash contents (Table 1). The presence of ash was indicator of amount of total solids. So the higher percentage of ash/total solids presented greater amount of contamination and vice versa [18].

3.1.2. Estimation of acidity

In the current study, the overall amount of acidity ranged from 2 to 9% (Table 1). The minimum concentration of acidity for plum sauce samples was noted as 2.43% while the maximum obtained value was 9.8%. Similarly, the mint sauce samples showed acidity concentration in range from 2.73 to 9.13% whereas for tomato sauce samples 2.56 to 9.63% range of acidity was observed. The higher acidity level was an indicator of less microbial growth while lower acidity concentration is considered as a promoting factor for microbial growth and may result in low quality of sauces for consumption [19].

3.1.3. Measurement of pH

The observations regarding pH of different sauces showed that maximum acidic value was obtained for sample of tomato sauce whereas maximum basic nature and statistically significant value at 0.01%

Table 1. Tabulated observations of physicochemical parameters sauce samples.

Samples	Ash content (%)	Acidity (%)	pH	Conductivity (mv/cm)	Sugar (%)
Plum	10.40 ± 2.04 (5)	5.26 ± 1.34 (5)	3.40 ± 0.04 (5)	22.33 ± 3.93 (5)	15.54 ± 2.05 (5)***
Mint	5.19 ± 1.07 (5)	6.98 ± 1.15 (5)	4.32 ± 0.07 (5)***	27.53 ± 3.73 (5)	3.35 ± 0.65 (5)
Tomato	7.61 ± 1.94 (5)	6.05 ± 1.28 (5)	3.68 ± 0.19 (5)	21.27 ± 6.64 (5)	6.05 ± 1.32 (5)

* All values are in mean ± SEM (n) and the results were found significance at 0.1% (***) level.

level was noted for mint sauce (Table 1). While the pH of plum sauces was in the range of 3.31 to 3.52. The detection of low pH was an indication of bacterial food contamination barrier but higher acidic pH of some sauce samples was also showing that these were already rich with microbial load, that's why; high concentration of hydroxide ions which occurred by high temperature. However; the sauce samples with high pH values were found more prone to microbial contamination [20].

3.1.4. Determination of electrical conductivity

The electrical conductivity reading was found maximum for mint sauce which was 27.53 mv/cm while minimum value of EC was 21.27 mv/cm recorded for tomato sauce (Table 1). The higher level of conductivity in eatables cause various diseases in humans like frequent urination, constipation, stomach pain, vomiting etc. whereas low level of electrolyte also results in such as thirst, fatigue, muscles weakness, loss of appetite, etc. That is why; standard values should be maintained but variations in electrical conductivity mainly occur due to the presence of impurities [5].

3.1.5. Determination of sugar percentage

According to the ANOVA results significant sugar content was found 15.54% in plum sauce at 0.01% level (Table 1) because addition of sugar is normally done to prepare this sauce and to reduce the microbial growth. Moreover, the brix test results for mint sauce showed minimum percentage of 3.35% which was an indication of no adulteration of any sugary content but of poor shelf life [10, 14].

3.1.6. Analysis of artificial sweeteners

In this test saccharine was detected at 217nm and aspartame was detected at 280nm. Saccharine and aspartame test had significant non polar characteristics because they had azo and aromatic rings. These artificial sweeteners are the alternative of sugar which is mostly added in the locally vended foods. Normal quantity of artificial sweeteners is safe to use but high quantity is harmful for the health of consumers [21].

In current study, aspartame was not found in any sample of sauces while for the saccharine detection, following results were obtained and in mint and some tomato samples, its adulteration was found which might be done to improve its taste and for better shelf life but it is considered harmful for health of consumers, if it is found more than 2.3 milligrams per pound and currently detected amounts were higher than standard value [22].

Table 2. Percentage of saccharine of plum, mint and tomato sauces.

Sample (s)	Observations
A, C, D, F, G, J, M	No saccharine
B	0.2872%
E	0.2221%
H	0.4271%
K	0.3463%
N	0.6267%
I	0.4258%
L	0.3706%
O	0.2967%

3.2. Microbial Analysis

3.2.1. Analysis of microbes by nutrient agar

For the detection of microbial contamination, first of all, general purpose medium (nutrient agar) was used and it was observed that maximum colony forming units were found in mint sauce which was 488 CFU/ml at 5% level of significance as compared to plum and tomato sauces, 407 CFU/ml and 360 CFU/ml, respectively. Whereas ANOVA results for the colony size were not found significant (Table 3). The probable identification showed that *Staphylococcus epidermidis, Bacillus simplex, Arthrobacter dextranolyticus, Pseudomonas cidrella* and *Bacillus weihenstephanesis* were present in currently studied local sauce samples (Figure 1). Moreover, the presence of *Staphylococcus epidermidis* showed the poor handling and hygiene practice by sellers which may result in food poisoning. Whereas the presence of *Bacillus simplex* indicated the prevailing improper storage practice and the detection of *Arthrobacter dextranolyticus* highlighted the usage of adulterants and poor grade ingredients. *Pseudomonas cidrella* indicated the sauce samples were not stored at ideal temperature and it is mainly transmitted in food items from infected hands of labor and handlers. Similarly, the presence of *Bacillus weihenstephanesis* was a clear indication of improper washing of used vegetables and fruits which were used to prepare sauces [25].

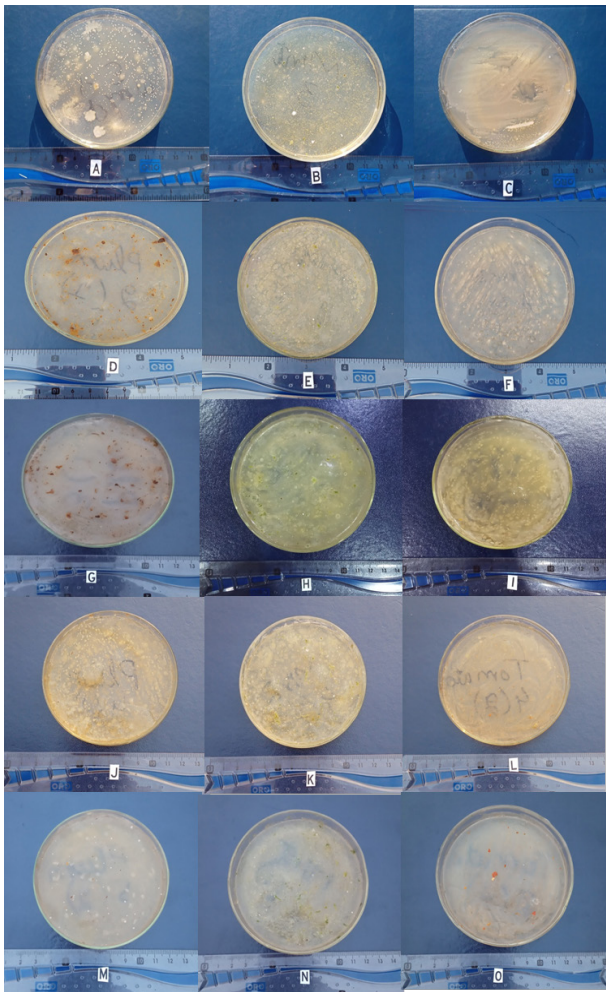

Fig.1. Morphological view on nutrient agar of microbial contamination detected in sauce samples and CFU/ml was calculated by using these plates: A, B, C, D, E (Plum sauce samples); F, G, H, I, J (mint sauce samples) and K, L M, N, O (tomato sauce samples).

3.2.2. Gram staining

All detected microbial strains were examined via gram staining to detect either they are gram positive or negative as confirmatory test. Gram positive bacterial contaminants were *Staphylococcus epidermidis, Bacillus simplex, Arthrobacter dextranolyticus* and *Bacillus weihenstephanesis* whereas *Pseudomonas cidrella* was gram negative [26].

3.2.3. Catalase test

The catalase test was also performed to detect catalase producers. Some samples including: A, D, E, F G, H and M, were catalase positive which indicated the presence of possible enteric bacterial

Table 3. Colonial diameter and CFU/ml on selected media for selected local sauces.

Sample	Microbial colonial diameter on nutrient agar (mm)	CFU/ml of nutrient agar	Microbial colonial diameter on EMB agar (mm)	CFU/ml of EMB agar
Plum	1.35 ± 0.47 (5)	407.4 ± 35.538 (5)	1.46 ± 0.17 (5)	472 ± 74.79 (5)
Mint	1.24 ± 0.21 (5)	488 ± 33.098 (5)*	1.76 ± 0.33 (5)	568.6 ± 74.66 (5)
Tomato	1.50 ± 0.59 (5)	360.8 ± 30.578 (5)	1.98 ± 0.43(5)	547.2 ± 108.12 (5)

* All values are in mean ± SEM (n) and ANOVA was applied and the results were found significance at 5% (*) level.

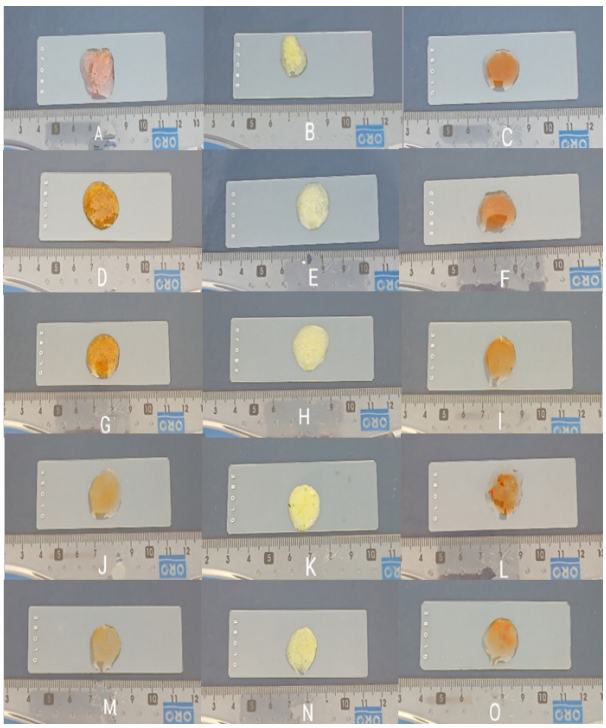

Fig. 2. Observations of catalase test. The samples A, D, E, F, G, H, M showed positive results whereas sample B, C, I, J, K, L, N, O were showed negative results.

strains while some samples including sample J, B, K, N, C, I, L and O were catalase negative which indicated their absence (Figure 2). Catalase test is performed only for currently obtained the gram positive bacteria. Samples which were catalase positive were enterobacteria [30]. The contamination of enterobacteria in food items like locally vended sauces occurs via skin, nails and GIT infections etc. of food handlers. But the major concern is presence of such pathogenic bacteria in food products may further cause several diseases such as infections of bones and joints [27].

3.2.4. Amylase test

The results of amylase test indirectly helped not only to detect type of microbial strains as contaminants but also indicated biochemical quality of sauces. The samples of sauces A, D, E, F, G, J, M, L and O showed positive results, i.e., microbial contaminants were not amylase producers so starch immediately appeared deep blue in culture plates while other samples of sauces B, C, H, I, K and N showed negative results in which starch was not found because color of iodine was not changed as starch content was already catalyzed by amylase producers (Figure 3). The presence of high level of

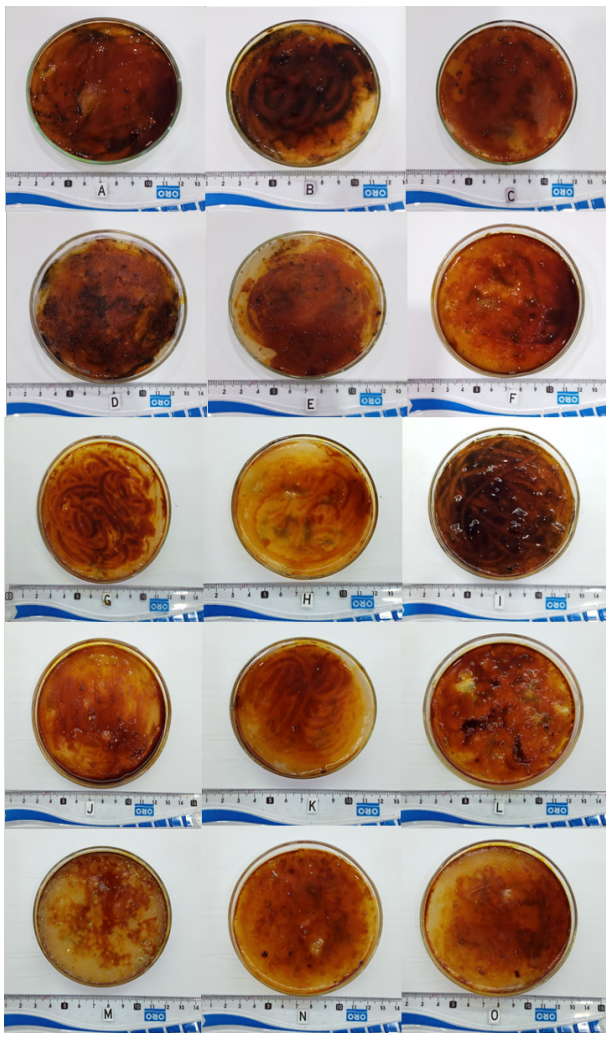

Fig. 3. Pictorial observations of amylase test for detection of starch. The samples A, D, E, F, G, J, M, L, O showed positive results while samples B, C, H, I, K, N showed negative results.

amylase in edibles than normal range may harm the health of consumers but still unchecked amount of starch is usually added by local venders in sauces to improve their consistency which results in more amylase production by microbes or contaminants because mostly these starch rich sauces are not stored according to the quality standards [23].

3.2.5. Coliform detection

To detect the microbial contamination of sewerage water (coliforms) selective medium, eosin methylene blue (EMB) agar was used and detected microbial strains included *Enterobacter aerogenus, Klebsiella pneumonia, Pseudomonas aerogenus, Escherichia coli* and *Proteus mirabilis* (Figure 4). The diameters of their colonies were measured

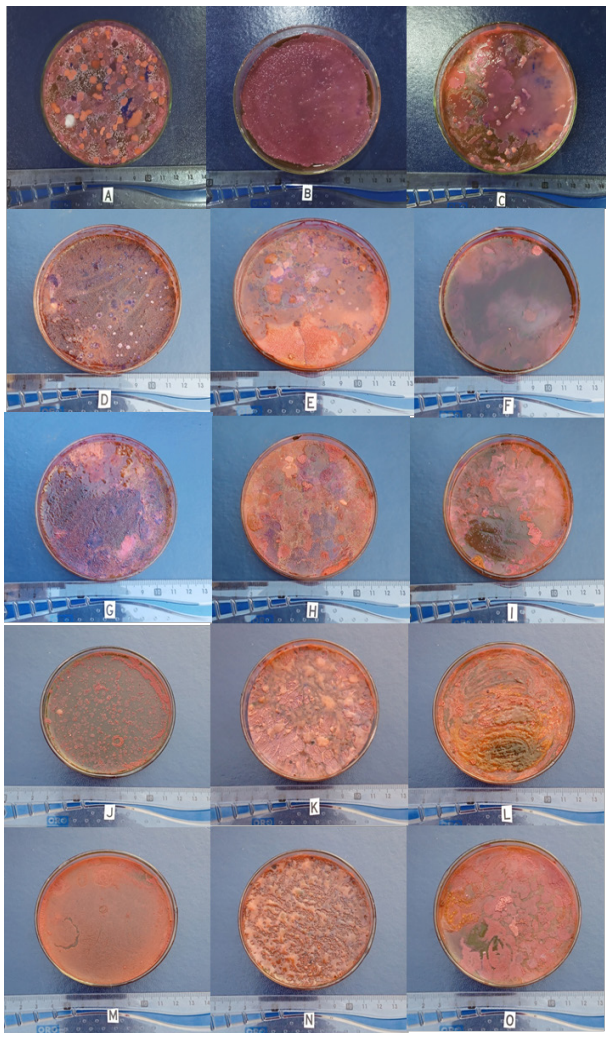

Fig. 4. Morphological view on EMB agar of coliforms contamination detected in sauce samples and CFU/ml was calculated by using these plates: A, B, C, D, E (Plum sauce samples); F, G, H, I, J (mint sauce samples) and K, L M, N, O (tomato sauce samples).

which were not found significant statistically. While the maximum coliforms were detected 568.6 CFU/ml in samples of locally vended mint sauce than plum and tomato sauces (Table 3). It means the used utensils, water added to sauces and hands of venders were might be contaminated coliforms either due to seepage of sewerage in to clean water supply which is utilized for dish washing and for preparation of local sauces or simply due to poor hygiene practice of venders [28]. Moreover, the intake of such coliform contamination having local sauces may result in skin, eye pneumonia, urinary tract and several other infections [24, 29].

4. CONCLUSIONS

It was concluded that among the selected commonly vended sauces, tomato sauce was overall more inappropriate for consumption than plum and mint sauces. Moreover, low grade ingredients were also used. As an outcome, consumption of these sauces may result in foodborne pathogenesis i.e., the way harmful ratio of artificial sweeteners was detected in tomato sauce. Similarly, contamination of several microbial pathogens was also detected in all selected samples of locally vended sauces. Thus organized steps of food authority of regular inspection and other public and private sector departments are required for the general public awareness and to train local venders about handling, preparation and storage of these sauces [31]. In this way, the quality of locally vended food items can be ensured.

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6. CONFLICT OF INTEREST

All authors have no conflict of interest.

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Research Article

Risk Factors Evaluation and Antiviral Eradication Therapies Among HCV Infected Family Members of Northern Regions, Pakistan

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Abstract: Hepatitis C virus (HCV) poses a global health threat, often transmitted intra-familial. This study aims to assess HCV transmission within Pakistani households and identify associated risk factors. To analyze 125 household subjects for HCV infection prevalence, demographics, genotypes, and risk factors. We conducted a comprehensive analysis of 125 household subjects to determine HCV prevalence. Demographics, genotypes, and risk factors were carefully examined through rigorous data collection and statistical analysis. Of the 125 households surveyed, 57 tested positive for HCV, indicating a high prevalence rate. Within these households, 121 individuals were infected, with a slightly higher proportion of females (59.5%). The distribution of infections varied across regions, with Islamabad showing the highest prevalence at 56.14%. Among the infected individuals, offspring were significantly affected (42.1%), followed by spouses (29.8%) and siblings (28%). Genotype 3a emerged as the most prevalent strain. Risk factors contributing to intra-familial transmission included major surgeries, dental procedures, hospitalizations, and blood transfusions. Furthermore, sharing personal items such as blades and towels also posed significant risks. Intra-familial transmission is a key driver of HCV spread within Pakistani households. The study's findings underscore the urgent need for targeted interventions to control and prevent HCV transmission within familial settings. Strategies should focus on raising awareness about risk factors and promoting preventive practices. Additionally, The most effective outcomes in the current study were observed with the combination therapy of Sofosbuvir and Ribavirin, achieving an End of Treatment Response (ETR) of 73% and a Sustained Virologic Response (SVR) of 72%.

Keywords: Intrafamilial Transmission, Household Subjects, Genotype, Therapeutic Regimens.

1. INTRODUCTION

Hepatitis is the inflammation of the liver, caused by HCV which was a non-A/non-B virus that was first discovered in the year 1989 and termed as Hepatitis C virus [1]. According to microbial taxonomy, HCV is categorized into the family *Flaviviridae* while its genera are *Hepacivirus*. It is a positive sense, single-strand RNA, enveloped virus. The size of the genome of this virus is about 9.6 kb which can encode only one protein after transcription, having the size of about 3000 amino acids. Both the necessary proteins for this virus, i.e., structural and nonstructural are present in this same protein which separated from each other after the translation process. Hepatitis virus C is further

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divided into six genotypes, with subtypes such as HCV 1(a) and (b) existing within each genotype [2]. Single protein that is encoded after the translation of the genome of this virus is cut into 10 different parts with the help of enzymes known as proteases. HCV contains protease enzymes for this purpose and also this virus takes the help of proteases from its host. The genome of this virus is translated in form "5UTR- C, E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B-3UTR". NS5B and NS2 stand for the non-structure proteins for this virus which helps in replication while E1, E2, and core-C are structural proteins. E1 and E2 proteins are present in the envelope of this virus [3, 4]. These structural and non-structural proteins are cleaved by peptides present in this virus which are named p7 [5]. Currently, there are 6main genotypes of the Hepatitis C Virus including several subtypes that have also been reported [6]. Geno-types are important that contribute to epidemiology, development of the vaccine, and diagnostic organization of chronic Hepatitis C disease [7]. Moreover, Hepatitis C genotypes are an important factor in the disease prediction for the constant response of virology [8]. These genotypes are significantly important that has a major influence on the course of infection and also on interferon therapy, as subject infected with different genotypes respond inversely to therapy.

The rate of prevalence of HCV in the whole world is in millions i.e. estimated about 170 M of people carry hepatitis, while the permanent carriers among them are 70 to 80 percent [9, 10].WHO has been reported that the prevalence rate of hepatitis C virus is 3 percent worldwide. Positive cases of hepatitis C are 170 million people around the world out of which the incident rate per year is 3 to 4 million while about 10 million HCV-positive cases are from our country [11]. Previous investigations reported the frequency rate of Hepatitis C with 1.6-1.8 percent and 1-2.3 percent in developed nations like America and Europe [12].

In the transmission of HCV to healthy individuals, some of them can eliminate the virus from the body while in the majority (from 30 to 60 percent), this virus leads to severe type of liver disorders like cancer and cirrhosis[13]**.** Some studies reported that transmission of HCV in the same family members mostly occurs through sexual contact between couple [14]. Some other studies also revealed other routes of transmission of HCV in family members like the use of the same materials used in the house such as utensils for food, towel, etc., [15, 16]. Still, the data regarded to the transmission of HCV in families is not sufficient to investigate the risk factors for intrafamilial clustering. Though transmission among families is not well investigated, various investigation indicates that perinatal and sexual transmission and close contacts as sources of contamination [17].

The diagnosis of HCV infection is carried out through serological assay and molecular assay [18]**.** The serological assay is involved in the identification of anti-HCV antibodies while the molecular assay is involved in the identification of HCV RNA in blood serum or plasma [19, 20]. Today certain antiviral drugs have been discovered to eradicate HCV infection, which are given in double or triple combinations. Drugs used in the double combinations are Peg-INFα + Ribavirin, Sofosbuvir + Ribavirin, Daclatasvir + Sofosbuvir, and Ledipasvir and Sofosbuvir. Ledipasvir and Sofosbuvir are also termed as harvoni therapy. These double combinations are given for 6 month time period. While the drugs in the triple combinations used for the treatment are Sofosbuvir + Peg-INFα + Ribavirin and Sofosbuvir + Daclatasvir + Ribavirin for the period of 3 months. Peg-Interferon alpha is given in the form of an injection to the patients to increase the immune response of the host against the virus present in the body by enhancing the activities of anti-inflammation and immune modulation. About one dose of 180 mg/ml of this injection is given in 7 days [21, 22].

2. MATERIAL AND METHODS

2.1. Samples and Data Collection

A total number of 125 families patients were enrolled having symptoms like pain in muscles and joints, dark urine, jaundice, fatigue, fever, abdominal pain, nausea, vomiting, and loss of appetite at the Department of Gastroenterology, PIMS, Islamabad, after diagnosed by a gastroenterologist. Before enrolment, they were also asked for any other HCV-positive patient in their family.

2.1.1. Inclusion criteria

Patients chronically infected with HCV have elevated Alanine aminotransferase (ALT) level in

the past six months 1.2 times greater than normal value, patient has positive anti-HCV antibodies result and positive PCR result for HCV RNA. The main focus of this was to enroll intrafamilial infected members.

2.1.2. Exclusion criteria

From the current study patients such as Patients ≤ 18 years, pregnant women, co-infected patients with HBV and HIV, breastfeeding mothers, liver cancer, and those patients who have already taken treatment for HCV infection were omitted.

2.1.3. Questionnaire

For obtaining patient history, the Questionnaire form was filled from patients at the Department of Gastroenterology, PIMS, and Islamabad. The questionnaire contains personal information such as monthly income, number of people in the family, gender, age, education, contact number, and number of rooms and also asked for family members infected with HCV. Patients having symptoms like abdominal pain, fatigue, fever, nausea, pain in joints and muscles, dark urine, jaundice, vomiting, and loss of appetite were enrolled in the current study. Furthermore, patients were asked for HCV transmission-related risk factors which include piercing through an instrument, IV cannulas, dental procedure, blood transfusion, surgical procedure, abscess treatment, five or more injections, endoscopy, colonoscopy, catheterization, sharing (blades, towel, toothbrush), shaving by barber, liver transplant, angiography, lithotripsy, exposure to HCV infected person and invasive process. Some additional questions such as abortion, five or more delivery, and traumatic delivery were asked from female patients in this study.

2.2. Antiviral Treatment for HCV

The patients were treated with a combination of five separate triple and double antiviral regimens after confirmation and detection of HCV genotype and viral load. Antiviral regimens such as 1. Sofosbuvir + Ribavirin 2.PegINF-α + Ribavirin 3.Sofosbuvir + Daclatasvir + Ribavirin 4.Sofosbuvir + Peg INF-α + Ribavirin and 5. Sofosbuvir + Daclatasvir. For HCV-infected patients, 180 mg/ml of injection (Peg-INFα) is advised once in seven days. Tablet Ribavirin (400 or 600 mg) recommend twice daily. Mechanism of action of ribavirin which is a guanine nucleotide analog that causes induce error during viral replication, inhibition of (IMPDH, immunomodulation) and direct inhibition of hepatitis C virus replication [23].

2.2.1. Adverse effects of antiviral therapies

Side effects of anti-viral therapies during the course of three to six months of treatment were noticed such as respiratory problems, thyroid dysfunction, gastrointestinal disorders, hematological, fatigue, depression, nausea, headache, and weight loss.

2.3. Diagnosis of HCV Infection

The ELISA-positive samples were further processed for RNA extraction and PCR analysis. Some samples were analyzed in accurate labs, RAWALPINDI while other samples were analyzed in the different laboratories of Islamabad.

2.3.1. Detection of viral load

In the present study, the load of HCV was categorized into different groups i.e., low, medium, marked, and positive. Patients with a load of < 100000 IU/ml were considered as low, while > 100000–500000 IU/ml and > 500000 were considered as medium and marked respectively.

2.3.2. RNA extraction

Total genomic RNA from a 150µL sample was extracted via viral nucleic acid extraction kit abott m2000sp (Abott Laboratories, USA) according to manufacturer protocol.

2.3.3. Reverse transcription PCR

For the detection of HCV's RNA in the specimen, initially through RT PCR the extracted RNA was reverse transcribed into cDNA using MMLV reverse transcriptase enzyme (Fermantas).

2.3.4. HCV genotyping

For genotyping, the virus was first amplified which requires 20 µl of the reaction mixture. This mixture also contained 10 µl of Green GO Taq Master Mix, 4 µl of cDNA, and forward and reverse primers in the quantity of 25 *p* mole. The process was completed in

40 cycles. These cycles were carried out at different temperatures, i.e., Denaturation was performed at 95 °C for 10 min, 95 °C for 1 min, annealing temperature was 55 °C for 1 min, amplification was performed at 72 °C for 1 min, and final refinement was performed on 72 °C for 10 min (Table 1) on GenAmpTM PCR system 9700 Applied Biosystems for amplification. Then gel electrophoresis was performed for the detection of PCR amplified product. Reverse and Forward primers such as KY80 = 5-GCAGAAAGCGTCTAGCCATGGCGT-3 and KY78 = 5-CTCGCAAGCACCCTA TCAGGC AGT-3 (Macrogen, South Korea) were used for both reverse transcription and PCR amplification in this study.

3. RESULTS

3.1. Prevalence of HCV

The prevalence of HCV among HCV-infected family members was detected as 45.6%. Prevalence of HCV-positive family members enrolled in this study was the resident of Islamabad 56.14%, KPK 14.03%, Punjab 28.07%, and AJK 1.75% as shown in Table 2.

3.2. Prevalence of HCV Infection In HCV-infected Families

The main focus of this study was to observe HCV infection among infected families. Family members were divided into three groups: (i) Parent's offspring, (ii) Spouses, and (iii) Siblings. High HCV infection was found in parent-offspring whose frequency was (42.11%), while in spouses (29.8%) and in siblings (28.07%) as shown in Figure 1.

3.3. Demographics' Socioeconomic and Education Level of HCV Infected Families Members

In this study, HCV-positive family-infected members were categorized into three age groups, ≥ 18-30, 31-50, and 51-80. The highest frequency of HCV was observed at 75.6% in 51-80 and 68.7% in 31-50 age group as compared to 44.9% in ≥ 18-30 group of HCV-infected familial HCV positive patients with a significance difference $p = 0.009$. The frequency of HCV among illiterates was high 56.5% as compared to literates 32.2% with a significant difference of $p = 0.007$. Prevalence of HCV among family members was observed in the low family income group (54.7%, 5000-20000) as compared to (25.7%, 21000-50000) and (25.0%, 51000-90000). A high prevalence of HCV among HCV-positive family members was observed in female patients at 61.5% as compared to male patients at 88.1% listed in the Supplementary Table 1. Mostly (39.4%, 6-10) number of patients enrolled in this study were living together in a (75.0%, 5-8) room home. Gender-wise HCV distribution is shown in Table 2.

3.3.1. Association of rrisk factors with HCV infection among HCV-infected families

A significant association of HCV infection was observed among HCV-infected family members

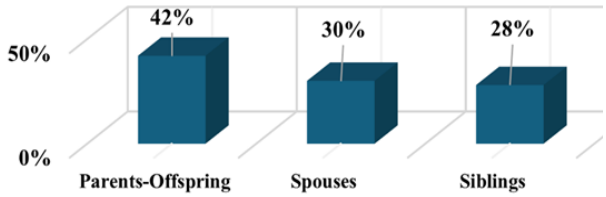

Fig. 1. Frequency of HCV in infected family members.

Table 1. Primers and Temperature for Amplification.

Target region	Product size	Primer Name and Sequence	Temperature
5′UTR	244bp	**Forward primer**	50°C: 2 min
		5-GCAGAAAGCGTCTAGCCATGGCGT-3	95°C:10 min
		Reverse primer	95°C:1 min
		5-CTCGCAAGCACCCTATCAGGCAGT-3	55°C:1 min
			72°C: 1 min
			72°C: 10 min

experienced with 5+ injection users $p = 0.000$, abscess-treated patients $p = 0.007$ and hospitalized patients $p = 0.004$, and insignificant association of HCV infection was observed among HCV-infected family members experienced with surgery $p = 0.510$, stitches $p = 0.459$, IV cannula $p = 0.433$, dental procedure $p = 0.278$, blood transfusion $p = 0.667$, dialysis $p = 0.400$, endoscopy $p = 0.534$, 5+ delivery $p = 0.145$ and traumatic delivery $p = 0.119$ listed in Table 2.

3.3.2. Risk factors associated with sharing of personal items

A significant association was observed between HCV-infected family members and patient exposure from infected persons $p = 0.000$ and facial threading $p = 0.029$. An insignificant association was observed with family members sharing personal items such as shaving by barbers sharing blades $p = 0.370$, sharing personal towels $p = 0.223$, and piercing by instrument $p = 1.170$ listed in Table 2.

3.4. Viral Load and Prevalence of Genotypes in HCV-Infected Family Members

The quantitively HCV RNA viral load was observed in HCV-positive family members and was categorized into three groups such as 10000-100000 IU/ml, 200000 - 500000 IU/ml, and ≥ 600000 IU/ml with 10.5%, 28.0%, and 61.4% respectively (Table 3). In this study, the most prevalent genotype 3a (98.2%) was detected followed by genotype 1 (1.2%) as shown in Supplementary Figure S1.

3.5. End-of-Treatment Response of Antiviral Therapies Among HCV Infection

End-of-treatment response (ETR) is the clearance of HCV virus infection after completion of three or six-month treatment of different combinations of double and triple antiviral regimens. The clearance of HCV virus after completion of antiviral treatment considered that ETR has been achieved. Five different antivirals were used in this study to observe ETR results. ETR was achieved in 02, 33, 1, and 6 HCV-positive family members treated with PegINF-α + Ribavirin, Sofosbuvir + Ribavirin, Sofosbuvir + PegINF-α + Ribavirin, Sofosbuvir + Daclatasvir and Sofosbuvir + Daclatasvir + Ribavirin therapy respectively (Figure 2). No ETR result was achieved in a patient treated with Sofosbuvir + PegINFα + Ribavirin therapy.

3.6. Sustained Virological Response of Antiviral Therapies Among HCV Infection

Sustained virological response (SVR) is defined as a viremia after six months or one year of the end of treatment response. In this study, SVR was achieved in 1, 21, 1, 2, and 4 treated with PegINF-α + Ribavirin, Sofosbuvir + Ribavirin, Sofosbuvir + Peg INF-α Ribavirin, Sofosbuvir + Daclatasvir, and Sofosbuvir+ Daclatasvir + Ribavirin therapy respectively (Figure 3). The most successful eradication regimen observed in this study was the Sofosbuvir + Ribavirin therapy.

3.7. Adverse Effects of Antiviral Therapies

More adverse effects were found in this study with Sofosbuvir+Ribavirin therapy. Side effects were also observed with other antiviral therapies used as discussed below:

3.7.1. Side effects of PegINFα + Ribavirin therapy

Mostly Side effects observed in 2, 1, 1, 1, and 1 HCV-positive patients treated with PegINFα+Ribavirin were hematological, dermatological, respiratory, and GIT disorders respectively.

3.7.2. Side effects of Sofosbuvir + Ribavirin therapy

More side effects found in HCV-positive individuals, who used Sofosbuvir+Ribavirin therapy for treatment in this study such as fatigue in 13, headache in 17, insomnia in 7, weight loss in 6, dermatological in 1, respiratory in 2 and GIT disorders in 2 patients were observed.

3.7.3. Side effects of Sofosbuvir + PegINF-α + Ribavirin therapy

Side effects observed with Sofosbuvir + PegINF-α + Ribavirin therapy in 1, 1, and 1 patients were headache, insomnia, and hematological disorders respectively.

3.7.4. Side effects of Sofosbuvir + Daclatasvir therapy

Adverse effects were detected when the patient

Table 2. Association of HCV infections with age, gender, surgical, non-surgical, and invasive procedures and sharing of personal items among HCV-infected families.

Risk factors (variables)	Infected family members	HCV infection (%)	OR (95%CI)	(*p*-value)
Gender				
Male	72	49 (68.1)	1.33 (0.71-2.47)	0.365
Female	117	72 (61.5)	1.00 (1.00-1.00)	1.000
Age				0.004
≥18-30	49	22 (44.9)	1.00 (1.00-1.00)	1.000
31-50	99	68 (68.7)	0.37 (0.18-0.75)	0.005
51-80	41	31 (75.6)	0.26 (0.10-0.65)	0.003
Surgery				0.510
Yes	50	29 (58.0)	0.45 (0.20-1.00)	0.510
No	75	28 (37.3)	1.00 (1.00-1.00)	1.000
Stiches				0.495
Yes	40	13 (32.5)	1.00 (1.00-1.00)	1.000
No	85	44 (51.7)	0.59 (0.20-1.00)	0.459
IV cannula				0.433
Yes	61	30 (49.2)	1.32 (0.65-2.68)	0.433
No	64	27 (42.2)	1.00 (1.00-1.00)	1.000
5+injections				0.000
Yes	34	3 (8.8)	1.00 (1.00-1.00)	1.000
No	91	54 (59.3)	0.06 (0.09-0.23)	0.000
Hospitalization				0.004
Yes	57	34 (59.6)	2.89 (1.39-6.00)	0.004
No	68	23 (33.8)	1.00 (1.00-1.00)	1.000
Dental procedure				0.278
Yes	57	29 (50.9)	1.48 (0.72-3.00)	0.278
No	68	28 (41.2)	1.00 (1.00-1.00)	1.000
Blood transfusion				0.667
Yes	24	10 (41.7)	1.00 (1.00-1.00)	1.000
No	101	47 (46.5)	0.82 (0.33-2.02)	0.667
Dialysis				0.400
Yes	4	1 (25.0)	1.00 (1.00-1.00)	1.000
No	121	56 (46.3)	0.38 (0.03-0.82)	0.400
Endoscopy				0.534
Yes	9	5 (55.6)	1.53 (0.39-6.02)	1.000
No	116	52 (44.8)	1.00 (1.00-1.00)	0.534
5+Delivery				0.145
Yes	6	01 (16.7)	1.00 (1.00-1.00	1.000
No	119	56 (47.1)	0.22 (0.02-1.98)	0.145
Traumatic delivery				0.119
Yes	2	2 (100)	0.96 (0.91-0.04)	1.000
No	123	55 (44.7)	1.00 (1.00-1.00	0.119
Shaving by barber sharing blades				0.370
Yes	12	04 (33.3)	1.00 (1.00-1.00)	1.000
No	113	53 (46.9)	0.56 (0.16-1.98)	0.370
Sharing personal towel				0.223
Yes	13	8 (61.5)	2.05 (0.63-6.66)	0.223
No	112	49 (43.8)	1.00 (1.00-1.00)	1.000
Piercing by instrument				1.170
Yes	60	23 (38.3)	1.00 (1.00-1.00	1.000
No	65	34 (52.3)	0.56 (0.27-1.15)	1.170
Facial threading				0.029
Yes	41	13 (31.7)	0.42 (1.93-9.25)	0.029
No	84	44 (22.4)	1.00 (1.00-1.00)	1.000
Exposure from HCV infected person				0.000
Yes	51	51 (100.0)	1.10 (0.04-0.22)	0.000
No	74	06 (8.1)	1.00 (1.00-1.00)	1.000

Table 3. Frequency of HCV viral load among HCV-infected family members.

S. No.	Viral load in IU/ml	Frequency	Percentage (%)
1	10000-100000 IU/ml	6	10.5
2	200000-500000 IU/ml	16	28.0
3	≥ 600000 IU/ml	35	61.4

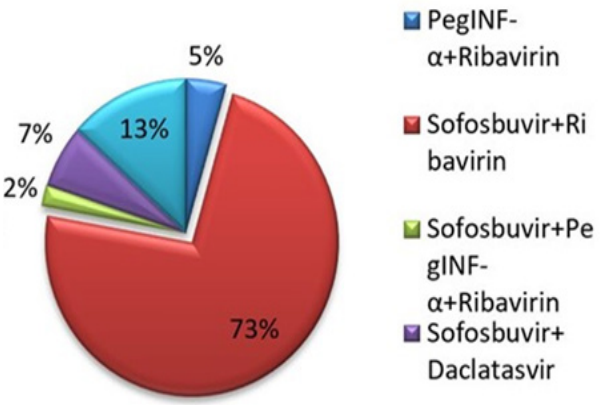

Fig. 2. ETR rates achieved with several antiviral therapies (in %).

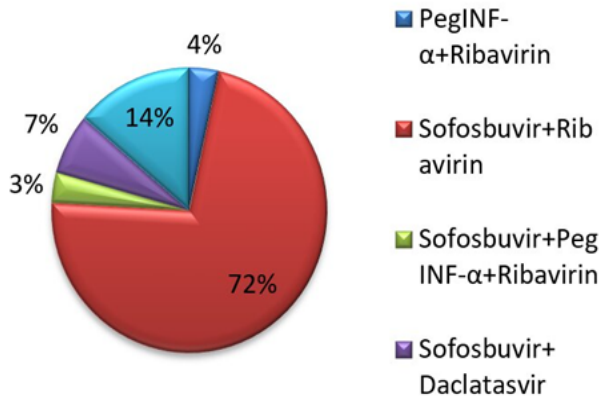

Fig. 3. SVR rates achieved with various antiviral therapies (in %).

treated with Sofosbuvir + Daclatasvir therapy such as fatigue, the headache were observed in 4 HCV-positive patients and insomnia in 1 HCV-infected patient.

3.7.5. Side effects of Sofosbuvir + Daclatasvir + Ribavirin therapy

Mainly observed side effects of Sofosbuvir + Daclatasvir + Ribavirin therapy in this study were Weight loss and dermatological adverse effects were observed in 2, respiratory and GIT disorders in 1, fatigue in 4, and headache in 5 patients.

4. DISCUSSION

HCV infects roughly 200 million individuals worldwide, with around 10 million (constituting 6.0% of the population) in Pakistan, creating a significant public health issue. The transmission of HCV within families affected by HCV has been hardly researched in the Pakistani community [24, 25]. Therefore, we aimed to investigate the specific aspects of HCV transmission, risk factors, anti-HCV treatment efficacy, and associated side effects in infected families who attended the Gastroenterology department at PIMS, Islamabad.

The prevalence of HCV in HCV-infected families (8.0%) was reported in Italy, and 7.0% in Spain [26, 27]. Another study revealed (16.2%) prevalence of HCV infection in households from Pakistan [28, 29]**.** In this study, a similar prevalence of HCV infection among family members was observed (45.6%) as compared to the previous study which is shown (44.2%) [30]. A high prevalence of HCV infection (56.14%) was observed in the population of Islamabad in this study. A previous study reported that the prevalence of HCV in infected families was (2.8%), (1.8%), (1.7%) and (2.3%) in mothers, fathers, siblings, and offspring respectively [31]**.** In this study, the prevalence of HCV infection was observed in parent-offspring (42.11%), in spouses (29.8%), and in siblings (28.07%). As compared to the previous study high prevalence of HCV among infected families was observed.

A previous study reported genotype 3a (89.0%) followed by genotype 3b (11.0%) in infected families member [28]. Qazi *et al.* [31] reported that genotype 3a (75%-90%) was prevalent in the Pakistani population. In this current study, the most prevalent HCV genotypes in infected families were genotype 3a (98.2%). That is similar to a previous study that strongly declared that genotype 3a is the main source of HCV infection in infected families. In this study, the risk factors that were most significantly found in HCV-infected families were 5+ injection users, hospitalized patients, and abscess-treated patients ($p = <0.05$), due to the reuse of contaminated syringes and needles, unhygienic condition of hospital and malpractices of clinical staff. An insignificant association of HCV infection was observed among HCV-infected family members experienced with surgery, stitches, dental

procedure, blood transfusion, dialysis, IV cannula, endoscopy, 5+Delivery and traumatic delivery (p = > 0.05). In this study sexual intercourse was not significantly important in the transmission of HCV to partners. Previous studies reported sharing of razors and toothbrushes are the main risk factor for the transmission of HCV among families as it is contaminated with a minute amount of blood and infected saliva or maybe from the carrier represents up to 20% of HCV positivity in the families [28].

Prevalence of HCV among family members was observed in the low family income group (54.7 %, 5000-20000) as compared to (25.7%, 21000-50000) and (25.0%, 51000-90000). Mostly HCV-infected families enrolled in this had (54.6%, 11-16) number of people living together in (75.0%, 5-8) rooms home. Unawareness and poverty are the factors of the spread of HCV among HCV-infected families. A previous study reported eradication rate ETR of Sofosbuvir + Ribavirin (99.4%), Sofosbuvir + PegINF-α+Ribavirin (93.3%), Sofosbuvir+ Daclatasvir (100%), Sofosbuvir+ Daclatasvir+ Ribavirin (86.6%) and PegINF-α+Ribavirin (70.0%) was achieved (Attiqullah, 2017). In the present study highly effective eradication (100%) ETR was achieved with each of Sofosbuvir +Ribavirin, Sofosbuvir+ Daclatasvir +Ribavirin, Sofosbuvir + Daclatasvir, and PegINF-α+Ribavirin therapy respectively [3].

In a previous study, SVR rates of 78.9%, 58.3%, and 100% were achieved when the patient treated with PegINF-α + Ribavirin, Sofosbuvir + Ribavirin, and Sofosbuvir +PegINF-α+Ribavirin respectively**,** and Successful SVR rate (95.4%), (100%) and (100%) was achieved with Sofosbuvir + Ribavirin, Sofosbuvir + Daclatasvir + Ribavirin, and Sofosbuvir +Daclatasvir respectively.

A previous study reported side effects observed when the patient treated with PegINF-α + Ribavirin therapy were fever (5.0%), headache (80.0%), fatigue (75.0%), insomnia (25.0%), and nausea (30.0%). In this study, similar adverse effects were detected such as fatigue (40.3%), headache (38.6%), and insomnia (15.8%) except fever (98.2%). In a previous study Patient treated with Sofosbuvir + Ribavirin had side effects of fatigue (75.0%), fever (4.8%), headache (75.7%), insomnia (27.0%), and weight loss (9.6%) **.**In this study side effects of Sofosbuvir + Ribavirin treatment were observed (22.8%), headache (29.8%), insomnia (12.3%), and weight loss (10.5%) [32].

5. CONCLUSIONS

Unawareness about HCV infection and transmission of HCV through sharing personal items, unsterile razors (shavers), toothbrushes, towels, and piercing noses and ears of many persons through contaminated needles or instruments at one time are risk factors for transmission of HCV in infected families. Awareness should be provided to the public for a healthy life. Higher authorities pay attention to overcome the threat of HCV infection through the provision of awareness and easily availability of anti-HCV treatment. Electronic and print media play their role in awareness about HCV infection and transmission. Academia and social media can play role in awareness about HCV infection and its mode of transmission among HCV-infected families.

6. ACKNOWLEDGMENTS

We confess the kind involvmemt of ethical committee and university for piloting this research graft.

7. ETHICAL APPROVAL

This study was conducted in PIMS and was approved by the ethics review board, PIMS, Islamabad, and the advanced study and research board of Abbottabad University of Science and Technology (AUST) KPK, Havelian. Patients were addressed about this study and consent was signed from them according to their well.

8. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Prevalence and Antimicrobial Susceptibility Patterns of Methicillin-Resistant *Staphylococcus Aureus* (MRSA) in Patients from District Bahawalpur

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Abstract: Methicillin-resistant *S. aureus (MRSA)* is an opportunistic bacterium causing a lot of infections, including infections of skin and soft tissues, endocarditis, pneumonia and bacteremia leading to death globally. This study aimed to determine the Methicillin-resistant *S. aureus* (MRSA) incidence and the antimicrobial pattern of MRSA among patients at a tertiary care hospital of Bahawalpur. Clinical samples (blood, sputum, catheter tips, pus, blood, urine, body fluid, wound, nasal and throat swabs) were collected from the hospital and transported to the microbiology laboratory. Culture and sensitivity testing was done in the laboratory to find out the antimicrobial susceptibility of *S. aureus* according to CLSI guidelines 2021. Among 622 collected specimens, 82 *S. aureus* isolates were found, and MRSA was found in 55 samples. MRSA was more prevalent in males as compared to females. In urine, a high incidence of MRSA was found. In age groups, a high prevalence was seen in the 61–70-year age group, and the lowest prevalence was seen in the 0–10-year age group. In OPD, the *S. aureus* prevalence was 39%. Among wards, the highest prevalence of MRSA was recorded in the ICU. Among *S. aureus* isolates, high resistance was shown by penicillin, cefoxitin and Sulphamethoxazole. Linezolid was found to be highly susceptible to *S. aureus* isolates. Linezolid, vancomycin, Fusidic acid, teicoplanin and clindamycin were the most effective antibiotics for treating infection caused by MRSA. The current study also noticed high resistance of bacteria to numerous antibiotics, revealing the significance of monitoring antibiotic consumption.

Keywords: *S. aureus,* MRSA, MSSA, Kirby Bauer, Antimicrobial Susceptibility.

1. INTRODUCTION

Staphylococcus aureus (S. aureus) is a well-known bacterium in humans, causing nosocomial infections in both community and hospital including skin and soft tissue infections, osteomyelitis, endocarditis, impetigo, and many other fatal diseases. It is a gram-positive, non-motile, spherical grape-like cluster and coccoid bacterium with a diameter of about 1 µm [1]. It is the common cause of death worldwide, indicating a poor diagnosis and treatment, having 20% to 40% fatality rate [2]. *S. aureus* is found asymptomatically on human body parts, i.e., skin and its glands, and mucous membranes, including healthy people's noses and guts as it can cause a variety of symptomatic infections, as it does not always live in harmony with humans [3]. In 1942, the first strain of *S. aureus* that was resistant to penicillin was discovered, and the penicillinase (β-lactamase) enzyme made antibiotics ineffective [4]. After methicillin was developed in late 1950, used to treat *S. aureus* infection, first methicillin resistant *S. aureus* was diagnosed in Europe in 1960 as it became resistant to β-lactam drug such as methicillin and amoxicillin [5]. The main cause of methicillin resistance is penicillin binding proteins (PBP2a) with low affinity, which is expressed by the mecA gene, on the staphylococcal

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chromosomal cassette mec (SCCmec) that results in resistance to antimicrobial β-lactams [6]. MRSA is classified into two types, community associated (CA-MRSA), and hospital associated MRSA (HA-MRSA) categories based on where the infection originated [7]. Individuals who have recently been hospitalized with serious diseases, the aged and those who reside in long-term healthcare facilities have more chances of HA-MRSA infections [8]. Health Care Workers (HCWs) are significant contributors to MRSA transmission in hospitals, as there are higher chances of colonization by continuous exposure to the hospital environment [9]. Virulent elements, including the toxic shock syndrome toxin 1(TSST-1), gamma-toxin, Panton Valentine leukocidin (PVL) and secretion of many exotoxins and enterotoxins by MRSA, cause infection such as cutaneous abscesses and mainly soft and skin tissue infection (SSTI), which if left untreated leads to critical diseases that may be fatal when CA-MRSA enters the cardiac, respiratory and skeletal systems. Due to increased virulence and transmissibility, CA-MRSA can spread terribly, whereas HA-MRSA cannot [10].

In Pakistan, the 1st MRSA case was spotted in 1989 [11], and its prevalence gradually increased [12]. In 2024, a 5.5% prevalence of MRSA was recorded in Faisalabad [13], and 89% was recorded in Lahore [14]. In Europe, the prevalence of infection caused by MRSA was found to be less than 5% in Finland, Netherlands, and Denmark while in Portugal and Malta, the incidence rate was more than 50% [15]. In Asian countries, the incidence of hospital and community linked MRSA was 67% and 25% respectively [16]. In the United States, there was a high rate of prevalence of about 60% in intensive care units with MRSA. About 80,000 infections were caused by MRSA with fatality rate of 14-20% annually [17]. Almost all strains of MRSA are highly resistant to various antimicrobial classes, including lincosamide, β-lactam drugs, aminoglycosides, and macrolides. Because of this, multidrug-resistant MRSA (MDR-MRSA) reduces the effectiveness of available treatments for *Staphylococcus* infections and causes severe outcomes, worsening the situation globally and becoming a severe health challenge for doctors [18]. Patients with staphylococcal infection are challenging to treat with the emergence of MRSA strains, lengthening hospital stays, increasing costs, and making them resistant to available antimicrobial drugs MRSA transmission and spread can be reduced and controlled by identifying health care workers (HCWs) who are colonized and are MRSA transmission vectors, using hand hygiene, and taking other preventive measures [19]. Unfortunately the decline in developing novel antibiotics in the face of quickly rising resistance to existing antibiotics exacerbates the situation. Due to resistance to glycopeptides, vancomycin has recently emerged as a source of worry, posing a problem in treating of tenacious MRSA infections [20]. Only a few antibiotics including Linezolid, Teicoplanin and Tigecyclines were effective against MRSA [21]. In recent years, the enormous incidence of MRSA and limited treatment options have emerged as quiet threats to public health. Therefore, this study aimed to investigate the methicillin-resistant *S. aureus*, its prevalence, demographic factors, and the antibiotic susceptibility of MRSA.

2. MATERIALS AND METHODS

This study was carried out at the pathology and endocrinology section of Quaid-E-Azam Medical College (QMC) and spanned from September 2022 to February 2023 and involved the collection of different clinical samples from the patients of the outdoor department (OPD) and indoor departments of Bahwal Victoria Hospital (BVH) using aseptic techniques. This study was conducted after approval from the Ethical Review Committee of our institution (letter No. IUB/ERC/25/2022), in 2022 and the simple random sampling technique was adopted to collect specimens according to the biosafety standards and international safety rules after informed consent to patients. Six hundred and twenty-two (622) different clinical samples, including blood, sputum, catheter tips, pus, blood, urine, body fluid, wound, nasal and throat swabs, were collected in highly sterilized, leak-proof, and dried containers under the supervision of medical officers using aseptic techniques before antibiotic use. From OPD, 73 samples were collected, and 61 samples were collected from the patients admitted in urology, nephrology, medical, surgical, ENT, paediatrics, paediatrics surgical, ICU, and CCU, respectively. Regardless of age and gender, patients belong to different regions of Bahawalpur and Bahwalnagar districts and was admitted or came to hospital were included in this study. From this study, patients who refused to participate and patients who experienced nasal bleeding were excluded because

rolling the swab could worsen the bleeding. To avoid duplication and respecting patient's privacy, a specific code was given to each sample.

Blood samples were collected in BACT/ ALERT blood vials and inoculate in an automated BACT/ALERT 3D for 5-7 days at 37 °C for microbial identification. Sterile swab sticks swab the wound and nose back into its case. All randomly collected samples were inoculated on different media, i.e., Blood, MacConkey and CLED (OXOID, UK), according to the sample type for culturing and incubated for 24 hours at 37 °C. After 24 hours, the bacterial growth on media was seen, and the identification of *S. aureus* was done by following standard identification procedures, including gram staining, the morphology of the colony, and biochemical test, i.e., catalase test, coagulase test and DNase test [22]. After confirmation, *S. aureus* was processed by using the Kirby-Bauer disc diffusion method for antimicrobial susceptibility testing [23]. Muller Hinton agar plates were used in antimicrobial susceptibility testing. Different types of antibiotics were used to test antibiotic sensitivity of *S. aureus,* including Penicillin (P) 10 µg, Cefoxitin (FOX) 30 µg, Erythromycin (E) 30 µg, Clarithromycin (CLR) 15 µg, Moxifloxacin (MXF) 5 µg, Teicoplanin (TEC) 30 µg, Sulphamethoxazole (SXT) 25 µg, Vancomycin (VA) 30 µg, Fusidic acid (FD) 10 µg, Linezolid (LNZ) 30 µg, Clindamycin (DA) 10 µg, and Tobramycin (TOB) 10 µg. On Muller Hinton agar, pure *S. aureus* isolated colonies were streaked, and antibiotic discs were impregnated, which were then incubated for 24 hours at 37 °C. After 24 h of incubation, the inhibition zone around the discs was measured using a scale and explained according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) [24] and Clinical and Laboratory Standards Institute (CLSI) recommendations [25]. Using Cefoxitin (FOX) 30 µg, *S. aureus* resistant to methicillin were identified with inhibition zone of 21 mm or less were MRSA while greater than 21 mm were considered MSSA. CLSI criteria from 2016 were used for teicoplanin, while results were demosntrated following the Clinical and Laboratory Standards Institute (CLSI) criteria from 2021. Categorical data were summarized using frequency and percentage using Microsoft Excel 365. The association between participant characteristics and *S. aureus* was analyzed using chi-square tests. A two-tailed method was employed with the significance threshold set at $\alpha = 0.05$. SPSS V.27 were used for statistical analysis.

3. RESULTS

3.1. Gram Staining

Under the light microscope, 82 (13%) isolates were found in Gram-positive cocci out of six hundred and twenty-two (622) specimens.

3.2. Biochemical Test

These gram-positive bacterial isolates were then confirmed for *S. aureus* by various biochemical test, i.e., catalase, coagulase and DNase test which showed positive results.

3.3. *S. aureus* Antibiotic Susceptibility

Among all (82) isolates, Linezolid was highly sensitive to 95%, while Teicoplanin, Vancomycin, Fusidic acid and Clindamycin were sensitive to 90% of all isolates. Penicillin was found to be highly resistant (76%). Cefoxitin was resistant to 67% of isolates, 62% resistant to Sulphamethoxazole, 59% to Clarithromycin, and 55% of all isolates were resistant to Erythromycin, Tobramycin, and moxifloxacin shown 34% and 21% resistance to isolates respectively (Table 1).

3.4. Prevalence of MRSA and MSSA

The prevalence of MRSA and MSSA was n = 55 (67%) and n = 27 (33%) respectively. Among the eighty-two (82) *S. aureus* isolates, 25 (31%) blood samples, 32 (39%) pus, 6 (7%) body fluids, and 19 (23%) urine samples were included. Out of 25 blood samples, 14 (56%) were MRSA, 11 (44%) were MSSA. Among 32 pus samples, 22 (69%) MRSA, 10 (31%) MSSA were included, six samples of body fluids included 4 (67%) MRSA, and 2 isolates of (33%) MSSA while 19 urine samples had 15 (79%) MRSA samples and 4 (21%) samples of MSSA were found. Among 82 *S. aureus* samples, 32 (39%) samples were from OPD and 50 (61%) samples were from different wards including urology, surgical ward, peads surgical ward, peads ward, medical ward, surgical ward, nephrology, ENT, ICU and CCU. From OPD, 21 (66%) isolates of MRSA and 11 (34%) isolates of MSSA were found. In urology, 87% (7) MRSA isolates were

Table 1. Antibiotic pattern of *S. aureus* isolates.

Classes of Antibiotics	Antibiotics	Resistant (%)	Sensitive (%)	P-value
Penicillin's	Penicillin (P)	62 (76)	20 (24)	0.305
Cephalosporins	Cefoxitin (FOX)	55 (67)	27 (33)	0.210
Macrolides	Erythromycin (E)	45 (55)	37 (45)	0.056
	Clarithromycin (CLR)	48 (59)	34 (41)	0.104
	Fusidic acid	9 (11)	73 (89)	0.438
Quinolones	Moxifloxacin (MFX)	17 (21)	65 (79)	0.353
Polypeptides	Teicoplanin (TEC)	8 (10)	74 (90)	0.448
	Vancomycin (VA)	8 (10)	74 (90)	0.448
Sulphonamides	Sulphamethoxazole (SXT)	51 (62)	31 (38)	0.150
Oxazolidinones	Linezolid (LNZ)	4 (5)	78 (95)	0.483
Lincosamide	Clindamycin (DA)	9 (11)	73 (89)	0.438
Aminoglycosides	Tobramycin (TOB)	28 (34)	54 (66)	0.210

found, 60% in surgical wards, 33% in peads surgical wards, and 42% in peads wards. MRSA was found more prevalent (100%) in nephrology, medical wards and in ICU respectively, followed by 75% in CCU and 0 isolate was found in ENT.

Out of 82 *S. aureus* isolates, 54 (66%) were male samples, and 28 (34%) were from female. The prevalence of MRSA among males was (n = 39) 72% and in females (n = 16), 57% of MRSA isolates were included. Conversely, MSSA was (n = 15) 28% prevalent in males and 43% (n = 12) in females. In different age groups, the frequencies of MRSA and MSSA was 35% vs 64% in 0-10 years, 57% vs 42% in 11-20 years, 63% vs 36% in 21-30, 55.5% vs 44% in 31-40 years age group. The highest prevalence of MRSA was seen in the 61-70 years age group, following 51-60- and 41-50-year age groups with frequencies of 88% and 80%, respectively . The data shows no significance for gender, specimen types, and collection site. Although, age may influence the *S. aureus* index with the p-value 0.013.

4. DISCUSSION

S. aureus is the primary cause of infections acquired in hospitals and communities worldwide due to its increased virulence and the ongoing emergence of antibiotic resistance. The last twenty years have seen an upsurge in the global spread of MRSA [26, 27]. In current research, the prevalence of *S. aureus* was 13% among collected samples, of which the MRSA and MSSA found 67% vs 33% prevalent. It is quite common in many nations, with prevalence rates greater than 80% in Latin America and rising to 19% in Australia [28]. In Pakistan, the prevalence of MRSA is increasing due to improper usage of antibiotics, as reported in the previous study, which was 78.3% in Hayatabad and 100% in Peshawar [29], 66% in Rahim Yar Khan [30, 31], and 52% prevalence of MRSA was reported in Karachi [32] and 56% was reported in a recent study [33]. The prevalence of MRSA was reported at 81% in hospitals in Egypt and 54.85% in Uttar Pradesh [34, 35]. In the current study, the prevalence of MRSA among *S. aureus* isolates was 67% which is close to the previous reported study. Among the total collected samples, the MRSA prevalence in the current study was 9%, which is agreed with the results of [21] in which a 10.4% prevalence of MRSA was reported and 6.37% in Peshawar [36]. The prevalence of MSSA in previous studies was 22% in Peshawar [31], and 35% in Islamabad [37], almost agreeing with the results of the current study in which the prevalence of MSSA was 33%.

The MRSA and MSSA prevalence in gender were studied, and in males, the prevalence of MRSA was higher than in females. The higher frequency of MRSA in males than in females was also reported by other researchers in Pakistan and India [21, 38-40]. A high (100%) frequency of MRSA was seen in the 61–70-year age group, followed by 88% in 51–60, 80% in 41–50, and the lowest prevalence was seen in the 0–10-year-old group, which was 35% in

Table 2. Detailed prevalence of *S. aureus* (MRSA & MSSA) among different age groups, specimen types, Gender-wise and in other Wards and OPD.

Variables	*S. aureus*	MRSA	MSSA	$\bar{X}$±SD	χ^2 (P-value)
AGE					16.213 (0.013)
0-10	17	6 (35.29)	11 (64.71)	2.69±2.897	-
11--20	7	4 (57.14)	3 (42.86)	15.71±3.302	-
21-30	11	7 (63.64)	4 (36.36)	26.45±2.733	-
31-40	9	5 (55.56)	4 (44.44)	34.88±2.315	-
41-50	15	12 (80)	3 (20)	47±1.732	-
51-60	17	15 (88.24)	2 (11.76)	55.58±2.526	-
61-70	6	6 (100)	0 (0)	68.33±3.777	-
GENDER					1.898 (0.168)
Male	54	39 (72)	15 (28)	27±16.97	-
Female	28	16 (57)	12 (43)	14±2.828	-
SPECIMENS					2.642 (0.450)
Blood	25	14 (56)	11 (44)	12.5±2.121	-
Pus	32	22 (69)	10 (31)	16±8.485	-
Fluid	6	4 (67)	2 (33)	3±1.414	-
Urine	19	15 (79)	4 (21)	9.5±7.778	-
WARDS & OPD					14.864 (0.095)
Urology	8	7 (88)	1 (13)	4±4.242	-
Surgical ward	10	6 (60)	4 (40)	5±1.414	-
Peads surgical ward	3	1 (33)	2 (67)	1.5±0.707	-
Peads ward	12	5 (42)	7 (58)	6±1.414	-
Nephrology	6	6 (100)	0	3±4.242	-
Medical ward	4	4 (100)	0	2±2.828	-
ICU	2	2 (100)	0	1±1.414	-
CCU	4	3 (75)	1 (25)	2±1.414	-
ENT	1	0	1 (100)	0.5±0.707	-
OPD	32	21 (66)	11 (34)	16±7.07	-

this research. A high frequency (100%) of MRSA was seen in the 61–69-year-old group in a previous study [31], 61%-68% was studied in different age groups [41]. The maximum prevalence of MRSA among isolates was seen in urine (79%) followed by pus (69%), fluid (67%), and blood (56%) in the current study. In Peshawar, the prevalence of MRSA in pus, fluid and blood was 35%, 42% and 48%, respectively [42], in blood, 100% MRSA prevalence was found [31] and 72% in pus samples [36]. In current study, the MSSA prevalence in blood was 44%, 31% in pus, 33% in fluid, and 21% in urine. The results reported from Narowal exhibit 53% prevalence in pus and 28% in blood [38].

S. aureus is responsible for 70% of ICU infections, many of which are MRSA [43]. The current study's prevalence was 100% in the ICU because of immune deficiency and serious diseased patient, who had more chances of infection. The

prevalence of MRSA in OPD was reported 63% in a study conducted in Rahim Yar Khan that closely agreed with this study's result in which the prevalence rate was 66%. In surgical ward, 39% prevalence of MRSA was reported. In different studies, the *S. aureus* resistance to penicillin was high (100%) but the findings of this study disagreed with the resistance exhibited by *S. aureus* to penicillin, which was 76%. Cefoxitin was found resistant in 67% of the isolates in this study which was in line with the results of study conducted in Rahim Yar Khan and Peshawar [30, 31]. Although, cefoxitin was 100% in some previous studies reported in different cities of Pakistan [32]. In Islamabad, cefoxitin resistance was low in compared to the present study [44]. Erythromycin was found resistant in 55% of *S. aureus* isolates. In previous studies, 99% and 84% resistance were documented in Peshawar [31, 42], 80.3% in Faisalabad [13], 78% in Rawalpindi [21], and 65% in Karachi, showing high resistance as compared to the present study [32]. Although low resistance of Erythromycin was also reported in some previous studies compared to the current study, 29% in Rawalpindi [41], and 46% in Rahim Yar Khan [30] of Pakistan. Clarithromycin was resistant to 59% of isolated strains in this study and was not correlated with the study conducted in Narowal and Peshawar [45, 46]. Fusidic acid resistance in the present study was lower as compared to the previous studies in Pakistan, revealing high resistance, 25% in Peshawar [31], 41% in Rahim Yar Khan [30], and 42% in Narowal [38]. In the current study the resistance of Clindamycin to MRSA isolates was 11%, which was not correlated with previous studies because of the resistance being too high. Clindamycin showed 51%-60% resistance to MRSA in previous studies from Pakistan [31]. In this study, Linezolid was only resistant to 5% of *S. aureus* strain and 95% susceptible to MRSA. In a former study conducted in Rawalpindi the Linezolid was 89.5% susceptible [21], and same trend was seen in [47]. Moxifloxacin was susceptible to 80% of isolates in a study conducted in Lahore [48]. The sensitivity of moxifloxacin to *S. aureus* isolates in the current study was noted 79% which was quite close to the findings of previous studies conducted in Lahore. The present study demonstrated that teicoplanin was susceptible to 90%. In Pakistan and Turkey, 100% susceptibility was observed in some previous studies [49, 50]. Vancomycin was susceptible to 90% of *S. aureus* isolates and resistant to 10%. The resistance in this study was a little higher than the previous study reported in Pakistan and different countries of the world. Vancomycin showed zero percent resistance and had outstanding results against MRSA in previous studies of Pakistan and India [36, 51]. In Islamabad, 97% sensitivity of Vancomycin was observed against MRSA [21], 95% in Iran [52] and 100% in Kabul [53] and 95% was reported in Ethiopia [54]. In our study, high resistance was shown by penicillin and cefoxitin subsequent to Sulphamethoxazole and macrolides.

The study explores important concerns in the healthcare field through the assessment of antibiotic resistance. However, it lacks the comprehensive classification of samples, and the lack of consideration of comorbidities as an aspect of risk factors. This study also does not utilize modern molecular methods of microbial analysis, such as whole-genome sequencing. Therefore, future studies should provide insight into the molecular mechanisms of resistance to fight against infections.

5. CONCLUSIONS

The rising trend of MRSA infections highlights the necessity of effective and strict infection control strategies. In the current study, over half (65%) of the isolates were MRSA, which was more prevalent in older adults and in males. Penicillin, cefoxitin, and sulfamethoxazole were highly resistant. Linezolid, clindamycin, fusidic acid, vancomycin, and teicoplanin were most susceptible in our research and can be used to treat infection caused by MRSA. Culture and sensitivity tests should be done to treat any infection before prescribing antibiotics and avoiding self-medication in order to control emerging resistance of microbes to commercially available antibiotics. Furthermore, research in different Pakistan regions is needed to gain insight into the epidemiology and molecular mechanisms of antibiotic resistance in MRSA.

6. ETHICAL STATEMENT AND PARTICIPANTS CONSENT

This study was approved by the Ethical Review Committee of "The Islamia University of Bahawalpur, Pakistan" under the letter No. IUB/ERC/25/2022. Written informed consent was obtained from all participants before their inclusion in the study.

7. ACKNOWLEDGEMENTS

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8. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Wheat Yield Gap Analysis: Productivity Enhancement Practices and Factor Level Categorizations

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Abstract: The demand for wheat is rising rapidly to sustain the growing population. In Pakistan, many farmers lack awareness of the optimal utilization of input factors. This study aims to determine the optimal use of these factors to enhance wheat productivity, address food security challenges and support effective policy decisions. The new concept for categorizations of agronomic factors is identified in current study as a high, medium and low loss factors to make accurate policy decisions for food sustainability. Statistical analysis is applied to 26430 crop cut experiments. The absolute and relative yield gap analysis is applied. Category-1 (major loss factors) refer to those factors, whose probability share (%) at optimum level lies in (1-25%) and these are urea (125-175) kg, DAP (100-125) kg, other fertilizers (25) kg and spray (2-3) for pest attack. The rest of the farmers are experiencing a decline in productivity. Category-II (Medium loss factors) refers those with a probability share of (26-50%) at optimum levels and these are 4 irrigations, harvesting between April 1st and 20th and certified seed (26.06%). Category-III (Minor loss factors) includes factors with a probability share of 51% or above at optimum levels such as November planting and soil type. A rise in the probability ratio of area share for categories I to III at their optimum levels results in enhancement in wheat productivity in diminishing order.

Keywords: Food Concerns, Wheat Yield Optimization, Yield Gap, Factors, Levels, Interactions.

1. INTRODUCTION

Agriculture is the biggest sector assuring food availability and straggling against food insecurity [1, 2]. It is projected that by 2050, the world's population will likely reach 9.1 billion and the main contributor to this increase will arise from developing countries. To feed the world, the production must be enhanced by about 70-100% [1, 3]. It is a human tragedy for us that each day our world witnesses 800 million individuals to go in hunger [4]. Despite the increase in world population, the growth rates of the yield for major cereals crops such as wheat, rice and maize are globally reduced [3, 5]. Wheat is the primary staple food crop in the world [6]. Pakistan is the 5th most populous country in the world with the highest population growth rate among South Asian countries. However, its wheat production is relatively low compared to other competing countries in the world [5, 7]. The agriculture sector of Pakistan is contributing about 18.9% share in the gross domestic product (GDP) and providing about 42.3% share of employment [8]. Wheat is the main food crop of Pakistan and is ranked first in acreage and production among all other food crops [9]. Pakistan stands 7th largest producer of wheat in the world [10]. Due to Pakistan's high population growth rate, it is emphasized that Pakistan will stand 4th populous state in the world instead of 5th by 2050 [1, 11]. In Pakistan, the population is growing rapidly, while the wheat crop yield is increasing at a slower pace and this is potentially creating a gap in meeting food requirements and raising the risk of food insecurity. Bajkani *et al.* [12] reported that applying traditional practices caused the Pakistan wheat crop's low productivity. Hussain [13] predicted that better inputs for food grain crop categories increasing return to scale for output. Tariq *et al.* [14] reported in Pakistan, the availability of wheat per capita will decrease from 198/kg in 2014 to 84/kg by 2050 due to the rising trend of population and adverse climatic variations. Abbas *et al.* [15] presented the factors liable for low wheat production and reported that

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poor management practices, weedicides, lodging, hailstorm etc., caused the low productivity of the wheat crop. Ali *et al.* [16] studied wheat production against different sowing times and concluded that the delay in sowing resulting a loss in productivity. Van-Ittersum and Cassman [17] presented the yield gap analysis to predict the need for food for the growing population and emphasized the yield gap analysis is one of the powerful measures to estimate production. Hatfield and Beres [18] depict that yield gap analysis is provide a framework and assessed the trend and yield difference of the wheat crop precisely. Van-Dijk *et al*. [19] presented the study on disentangling agronomic and economical yield gap and reported that the frame work of yield gap analysis is practically operational.

In Pakistan, wheat productivity is low compared to competitive countries, while the population growth rate is high, which may lead us to a food-insecure region. It is necessary to elaborate on the factors levels and their interactions that may statistically optimize the wheat productivity. Several studies were carried out in Pakistan on the individual contribution of inputs. At the same time, it is important to know that factors like fertilizers, pesticides, sowing period, harvesting periods, seed quantity, seed source, irrigations etc., applied at their various levels and have their individual and interaction effects on wheat productivity. The current study focuses on the individual and interaction study of these inputs at different levels responsible for enhancing wheat productivity. Moreover, in south Asian countries like Pakistan, maximum farmers are low educated or illiterate and they are applying different agronomical constrains in field experiments. They are not aware about the optimum level of factors which can maximize the productivity. This research gives the direction to those low or illiterate formers for using the optimum level of factors. At the same time this study introduces the new concept for categorizing agronomic constraints into high, medium and low-risk (loss) factors, which leads to the layout of the true policy's decision to attain food sustainability. It is based on statistical analysis to elaborate on the optimum level of wheat productivity with the application of various inputs and their interaction levels. The absolute and relative yield gap analysis is also emphasized to elaborate on the yield loss for different input levels. The significance of the results is assessed through statistical analysis.

2. MATERIALS AND METHODS

2.1. Data Source, Validity and Sampling Techniques

According to the area, Punjab is 2nd largest province of Pakistan. Punjab is responsible for 76% share of the total wheat area [20]. For the present study, secondary data of 26430 crop cut experiments (CCE) is collected from the Crop Reporting Service (CRS), Agriculture Department Punjab from 2017 to 2020. The CRS is the only statistical organization working independently since 1978 and is responsible for forecasting the area, yield, and production of all major and minor crops [20, 21]. The estimates of CRS are further published by the Bureau of Statistics (BOS), Punjab, Pakistan Bureau of Statistics (PBS) and many other government agencies for the researchers and policymakers. Table 1 shows the sampling design for area frame sampling. In stage-I, probability proportional to size (PPS) is used to select the sample village and in stage-II, Simple Random Sampling (SRS) is used to select the village (segments) [20-22].

2.2. Factors Levels and Statistical Analysis

The average yield mound (mds)/acre of crop cut experiments in Punjab is taken as a dependent factor

Table 1. The 2nd stage area frame sampling design.

Area Frame Sampling Design		
Stage-I	Sampling Technique	(Probability Proportional to Size sampling (PPS)
	Union Council (UC)	Population
	Villages	Sampling Unit
Stage-II	Sampling Technique	(Simple Random Sampling (SRS)
	Village	Population
	Land Segment	Sampling Unit

and the effects of following variables and their levels are studies as agronomic constrains (Table 2). Descriptive statistical analysis is presented with tabulation and graphical presentation of individual factors with their levels and interaction effect of factors with their levels. The significance of the groups (levels) means differences is testing using analysis of variance (ANOVA). Normality of the data is a fundamental assumption in applied data analysis. For the current large dataset, the most effective approach to evaluate and interpret normality is through graphical methods using histograms with a normal curve and Q-Q plots [23, 24]. Statistical tools are used as mean yield, relative frequency (R.F), absolute yield gap (loss) and relative yield gap (loss) etc. The following yield gap analysis is applied.

I. The absolute yield gap analysis is applied to check the yield loss in absolute term.

$$Abs\bar{Y}_{igap} = \bar{Y}_{i(op)} - \bar{Y}_{i(ay)} \quad (1)$$

II. The relative yield gap analysis is employed to determine the percentage loss in productivity.

$$Rel\bar{Y}_{igap} = [\frac{\bar{Y}_{i(op)} - \bar{Y}_{i(ay)}}{\bar{Y}_{i(op)}}] * 100 \quad (2)$$

Where "i" = individual levels of "ith" factor, "op" = optimum (maximum) average yield of "ith" factor at a specific level, "ay" = average yield of "ith" factors levels.

2.3. Concept of Categorization of Agronomical Constrains

Wheat yield varies by changing the level of inputs factor. At some level of inputs factors, the wheat yield found optimum, while on the rest of inputs levels, the yield reduced on some or more extends. However, the probability share (%) of the farmers using different level of inputs also various from one level to another level. Categorization of variable is applied in term of probability share (%), yield gap analysis and productivity.

I. Major loss factors mean factors, whose probability share (%) at optimum level of inputs lies in 1-25%, while the rest of the farmers are applying non-optimum level and getting loss in productivity.
II. The medium loss factors mean factors, whose probability share (%) at optimum level of inputs lies in 25.1-50%, while the rest of the farmers are applying the non-optimum level and getting loss in the productivity.
III. Minor loss factors mean factors, whose probability share (%) at optimum level of inputs lies in 50.1% and above, while the rest of the farmers are applying the non-optimum level and getting loss in the productivity.

2.4. Analysis of Variance (ANOVA) for the Significance of Mean Difference

ANOVA is applied to compare the significance mean differences in the groups of variables. F-Statistic is applied to investigate whether or not the groups are statistically differing or not. The following statistical hypothesis is examined:

H_0: There is no significant difference between the means of factors (variables) groups.
H_1: There is significant difference between the means of factors (variables) groups.

Table 2. Factor levels of agronomic inputs.

Input factors	Factor levels
Planting period	October 15-31, November 1-15, November 16-30, December 1-15, December 16-31, January 1-15
Harvesting period	April 1-10, April 11-20, April 21-30, May 1-15
Fertilizers (DAP, Urea, Other fertilizers	0 kg/acre, 25 kg/acre, 50 kg/acre, 75 kg/acre, 100 kg/acre, 125 kg/acre, 150 kg/acre, 175> kg/acre
Irrigation/ No. of water	(0-9) irrigations
Seed type	Certified or un-certified
Soil type	Chikny soil and other type (sandy etc.)
Pest attack	Pest attack yes or no
Pest spray	Pest spray (0-3)

2.5. Limitations of the Study

The article addresses a critical issue of global significance. The innovative approach of categorizing agronomic factors into high, medium, and low risk groups provides actionable insights for policy formulation. The focus on optimizing input levels and identifying key yield gap contributors is highly relevant, particularly for South Asian countries like Pakistan. However, certain key limitations are associated with this study. This research did not emphasis on the individual yield of the specific farmers located in different region of Punjab. It only focuses on over all Punjab average yields in mds/acre obtained from crop cut experiments and it may not fully capture the dynamic variability of field conditions across different regions in Punjab. The findings may not be directly applicable to farmers with diverse socio-economic backgrounds or varying levels of access to resources. This research focuses on certain levels and categorizations of factors. However, there is a need for more intricate levels and categorizations of agronomic factors in field experiments. The research does not account for potential climate change impacts on wheat productivity and the applicability of the identified optimal levels under changing environmental conditions. The study emphasizes statistical analysis while excluding advanced techniques such as supervised and unsupervised machine learning methods.

3. RESULTS

3.1. Normality Analysis

It is evident from Figures 1 and 2, that the normality through histogram and Q-Q plot of wheat crop productivity exhibits a strong pattern of normality.

3.2. Wheat Productivity in Response of Planting and Harvesting Periods

In Punjab, wheat crop sowing starts in late October and ended up by mid- January. From Table 3, the disparity in average wheat productivity was observed for various levels of planting periods. The maximum average yield in Punjab is 36.76 mds/acre for the best planting period, which is the first fortnight of November, while for the second fortnight, it is 36.46 mds/acre with an absolute yield loss of 0.30 mds/acre and relative loss of 0.82%. The lowest productivity is observed for October sow, with an absolute yield gap loss of 12.31 mds/acre and relative yield loss of 33.48%. Fortnights of December exhibit average yield gains of 34.30 mds/acre and 32.75 mds/acre with an absolute yield gap of 2.46 mds/acre and 4.01 mds/acre having percentages of 6.70% and 10.91%. For January and onward, the absolute yield gap found 10.03 mds/acre. The maximum wheat area is sown in the second fortnight of November at about 53.20%, while the optimum level of output gained for the first fortnight of November, having sown area share of about 33.83%. The wheat gains good productivity for the sow of November.

The wheat harvesting period prevails in Punjab from April to May. Table 3 shows the variation in output in response of harvesting periods. The optimum level of average productivity of 39.94 mds/acre is attained for harvest of April (1-10). The

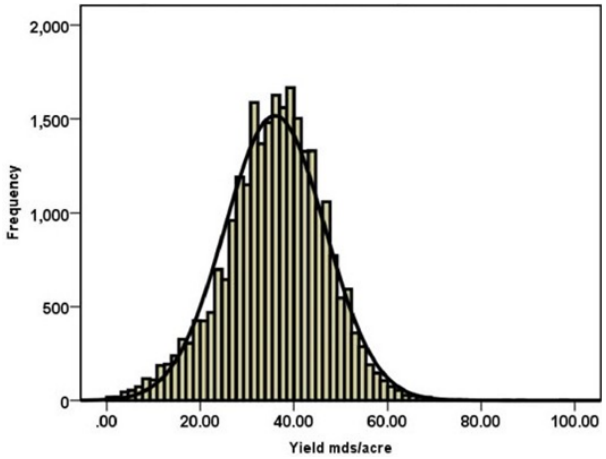

Fig.1. Histogram for the wheat yield.

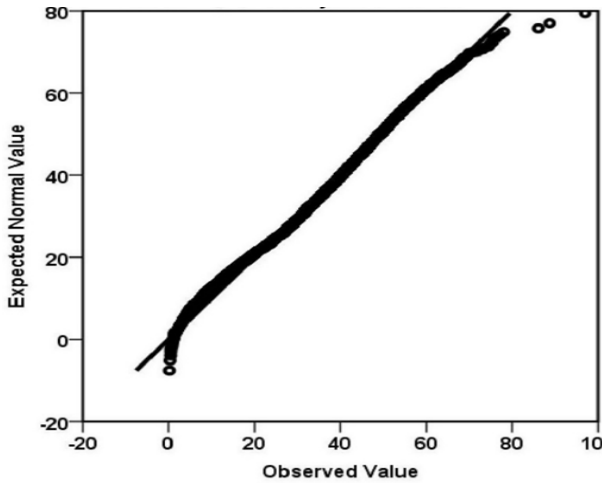

Fig.2. Q-Q plot for the wheat yield.

loss in average yield is noted for April (11-20) is 2.45 mds/acre and 6.13% and for April (21-30) is 5.15 mds/acre and 12.89%. The harvest period of May (1-15) depicts an average yield loss of 10.44 mds/acre and 26.13%, and for the last fortnight of May, it is 18.74 mds/acre and 46.92%. Delay in harvesting resulting higher the yield loss while planting other than in November, resulting in a loss in productivity.

3.3. Interactions Study for the Planting and Harvesting Periods

From Table 3, it is clear that the wheat crop was planted from the end of October and ended up to start January, and its harvesting started from the start of April and ended in May. The optimum wheat output must depend upon the period required to mature the crop from sown to harvest. The interaction effects of planting and harvesting periods are proposed and need to be elaborated statistically to determine the optimum output level. Table 4 shows the disparities in wheat productivity in response to the interaction effect of different sowing and harvesting periods. It is predicted that the optimum productivity gained for harvest periods of April and planting periods of November. It indicates that the wheat crop should be sown at the beginning of November and harvested between April 1st and 20th. Sowing the wheat crop in October, December and January leads to an average productivity loss.

3.4. Wheat Productivity in Response of Different Fertilizers Levels

Optimum level of different fertilizers is a yielding characteristic for wheat productivity, but excess fertilizer may lose productivity. The most prominent fertilizer in Pakistan is Diammonium phosphate (DAP) consists of nitrogen 18% and phosphate 46%,

Table 3. Wheat average productivity disparity with varies in levels of planting and harvesting period.

Planting period	R.F. (%)	Yield mds/acre	Abs. gap	Rel gap (%)	Harvesting period	R.F. (%)	Yield mds/acre	Abs. gap	Rel gap (%)
Up to Oct 31	3.14	24.45	12.31	33.48	April 1-10	6.67	39.94	0.00	0.00
Nov 1-15	33.83	36.76	0.00	0.00	April 11-20	37.80	37.49	2.45	6.13
Nov 16-30	53.20	36.46	0.30	0.82	April 21-30	51.38	34.79	5.15	12.89
Dec 1-15	7.56	34.30	2.46	6.70	May 1-15	3.96	29.50	10.44	26.13
Dec 16-31	2.06	32.75	4.01	10.91	May 16>	0.17	21.2	18.74	46.92
Jan 1-onward	0.22	26.73	10.03	27.29	--	--	--	--	--

Table 4. Interaction study of planting and harvesting period for wheat crop productivity.

Planting periods	Harvesting period	Yield mds/acre	Abs. gap	Rel gap (%)	R.F. (%)	Planting periods	Harvest period	Yield mds/ acre	Abs gap	Rel gap (%)	R.F. (%)
Up to Oct 31 3.14(%)	April 1-10	37.84	0.00	0.00	0.17	Dec 1-15 7.56 (%)	April 1-10	38.07	0.00	0.00	0.50
	April 11-20	25.91	11.93	31.52	0.75		April 11-20	35.94	2.13	5.59	2.78
	April 21-30	23.52	14.32	37.84	1.86		April 21-30	33.42	4.65	12.22	3.86
	May 1-15	20.78	17.06	45.09	0.34		May 1-15	27.69	10.38	27.26	0.38
	May 16>	9.80	28.04	74.10	0.03		May 16>	10.50	27.57	72.43	0.02
Nov 1-15 33.83 (%)	April 1-10	40.97	0.00	0.00	3.09	Dec 16-31 2.06 (%)	April 1-10	37.12	0.00	0.00	0.12
	April 11-20	37.88	3.09	7.54	13.33		April 11-20	35.88	1.24	3.34	0.64
	April 21-30	35.56	5.41	13.20	16.25		April 21-30	31.83	5.29	14.24	1.12
	May 1-15	30.38	10.59	25.84	1.09		May 1-15	23.78	13.34	35.94	0.17
	May 16>	14.54	26.43	64.51	0.06	Jan 1-onward 0.22 (%)	April 11-20	25.48	4.05	13.72	0.02
Nov 16-30 53.20 (%)	April 1-10	39.39	0.00	0.00	2.78		April 21-30	29.53	0.00	0.00	0.14
	April 11-20	37.93	1.46	3.70	20.28		May 1-15	19.11	10.42	35.30	0.05
	April 21-30	35.45	3.94	10.00	28.14		May 16>	25.68	3.85	13.05	0.00
	May 1-15	31.66	7.73	19.61	1.93		--	--	--	--	--
	May 16-onward	34.51	4.88	12.40	0.07		--	--	--	--	--

and urea contains phosphate 46%. In Punjab, some farmers supplement their use of DAP and urea with other fertilizers such as Ammonium Nitrate, Single Superphosphate (SSP) and Nitrofast. Table 5 depict the different levels of DAP, urea and other fertilizers used to gain optimum output. Fertilizers DAP and urea is available in 50/kg bags. For the urea, the maximum output is gained at between (125-175) kg/acre. In contrast, the farmer's majority uses the 100 kg/acre urea with a probability ratio of about 47.9% and losses the production of about 3.45 mds/acre and 8.29% compared to 175 kg/acre. The no use of urea resulted in 51.58% yield loss, while for half bag of urea it is 38.49%, compared to the optimum output level. The DAP gained maximum output at 100 kg/acre (2 bags), but only 4.40% of farmers use this DAP level. 81.4% of farmers use 50 kg/acre (one bag) and get a loss of productivity of about 6.03 mds/acre. The 41.43% yield losses to those farmers who are not using the DAP. In the case of other fertilizers, 92.3% of farmers did not use other fertilizers and lost the average productivity about 14.86%, while the others applied the other fertilizers with or without DAP and urea. The optimum level of the other fertilizers is 25 kg/acre with DAP and urea application and for upper levels of other fertilizers, the productivity losses are 8.75%, 4.84%, 6.49%, and 11.57%. It is clarified that the optimum level of DAP is up to (100-125) kg/acre, and below that level, the productivity gradually losses, while for urea, the good level is (125-175) kg/acre and below that levels productivity gradually losses.

3.5. Interactions Study for Different Fertilizers Levels

Fertilizers DAP and urea is commonly used in Pakistan. The interaction study for different levels of DAP and urea is critiqued in Table 6. It interacts that for the level at no use of DAP, the urea predicted almost optimum level at 125 kg/acre with 46.41 mds/acre followed by 42.43 mds/acre at 175 kg/acre urea. For 25 kg/acre use of DAP, the wheat crop predicted a maximum output at 125 kg/acre urea of about 40.69 mds/acre. The yield is good for 50 kg/acre DAP at (125-175) kg/acre urea, followed by lower urea levels. At 75 kg/acre application of DAP, the productivity maximized at 46.37 mds/acre for > 175 kg/acre urea, followed by 125 kg/acre at 44.85 mds/acre and 42.63 mds/acre for 150 kg/acre. With the 100 kg/acre used of DAP, the yield is almost attaining maximum at (125-175) kg/acre urea level. The highest optimum yield level is 51.12 mds/acre attained for > 125 kg/acre DAP with 100 kg/acre urea, and at this level of DAP, the yield is followed by a lower level of urea, while decreasing for a higher level of urea.

3.6. Wheat Productivity in Response of Irrigation, Certified Seed and Soil Type

Irrigation is a productive part of wheat productivity. Table 7 indicates four irrigations is optimum level, where the wheat crop yields maximum productivity of 39.17 mds/acre. About 27.82% of farmers are applying the optimum level of irrigation in Punjab, while the rest are not applying the optimum level and get a yield loss of about 52.0% for no irrigation, 22.08% for one irrigation, 8.42% for two irrigations, 3.19% for three irrigations, 3.42% for five irrigations, 13.68% for 6 irrigations and this gap is increasing up to 23.31% for 9> irrigations. The seed is an important part of better productivity. The analysis in Table 7 predicted that only 26.06%

Table 5. Different fertilizers, their levels and wheat productivity.

Fertilizers levels in kg	Urea				DAP				Other fertilizers			
	R.F. (%)	Yield mds/acre	Abs. gap	Rel gap (%)	R.F. (%)	Yield mds/acre	Abs. gap	Rel gap (%)	R.F. (%)	Yield mds/acre	Abs. gap	Rel gap (%)
0	3.29	20.15	21.47	51.58	4.91	24.59	17.43	41.43	92.3	35.69	6.23	14.86
25	1.14	25.60	16.02	38.49	2.12	26.57	15.5	36.84	0.64	41.92	0.00	0.00
50	30.2	32.86	8.76	21.04	81.4	36.04	6.03	14.33	5.62	38.25	3.67	8.75
75	10.1	37.47	4.15	9.97	7.07	41.37	0.70	1.66	0.13	39.89	2.03	4.84
100	47.9	38.17	3.45	8.29	4.40	42.07	0.00	0.00	1.08	39.20	2.72	6.49
125	2.06	41.50	0.12	0.29	0.04	41.83	0.24	0.57	0.20	37.07	4.85	11.57
150	4.87	40.01	1.61	3.87	--	--	--	--	--	--	--	--
>175	0.37	41.62	0.00	0.00	--	--	--	--	--	--	--	--

Table 6. Interaction study for optimum productivity at different levels of DAP and urea.

DAP	Urea	Freq	Yield mds/acre	DAP	Urea	Freq	Yield mds/acre	DAP	Urea	Freq	Yield mds/acre
0	0	396	16.17	25	0	69	16.18	50	0	375	23.72
	25	28	21.36		25	114	24.32		25	147	25.68
	50	542	25.65		50	212	25.67		50	6684	33.14
	75	69	34.20		75	68	33.76		75	2167	37.10
	100	227	32.44		100	84	32.82		100	10797	37.80
	125	7	46.41		125	9	40.69		125	365	40.0
	150	27	33.43		150	4	32.43		150	923	38.88
	>175	1	42.43		>175	--	--		>175	72	40.0
75	0	14	34.30	100	0	14	38.99	>125	0	2	47.07
	25	11	45.55		25	2	43.89		25		--
	50	273	38.54		50	278	40.12		50	1	47.17
	75	304	40.77		75	67	41.55		75		--
	100	985	41.76		100	567	41.93		100	3	51.12
	125	116	44.85		125	44	45.31		125	3	25.78
	150	162	42.63		150	172	44.80		150		--
	>175	5	46.37		>175	19	46.08		>175	1	46.25

Table 7. Irrigations, certified seed, soil type and wheat productivity.

Factors	Levels	R.F. (%)	Yield mds/acre	Abs. gap	Rel gap (%)
Irrigations level	0	5.74	18.80	20.37	52.00
	1	8.15	30.52	8.65	22.08
	2	15.15	35.87	3.30	8.42
	3	26.82	37.92	1.25	3.19
	4	27.82	39.17	0.00	0.00
	5	10.14	37.83	1.34	3.42
	6	4.25	33.81	5.36	13.68
	7	1.30	30.63	8.54	21.80
	8	0.39	29.58	9.59	24.48
	9>	0.23	30.04	9.13	23.31
Certified seed	Yes	26.06	37.27	0	0
	No	73.94	35.45	1.82	4.90
Soil type	Chikny	66.30	36.88	0	0
	Others	33.70	34.05	2.83	7.67

of farmers are using certified seed in Punjab and getting maximum output compared with 73.94% of farmers who are debarred from certified seed and getting yield loss of 1.82 mds/acre. The soil type (chikny) produced productivity of 36.84 mds/acre with a relative share in the area is 66.30%, and other types of soil (sandy etc.) reported yield loss of 7.67%, having a share of 33.70%.

3.7. Interactions Study of Pest Attack and Spray Operations

Pest attack is a common phenomenon for the agriculture sector in Pakistan and the world, which have become the case of losing productivity. The interactions study for the pest attack with its eradication through a spray operation shown in

Table 8. It interacts that 75.2% of farmers are those who lost a loss of 3.82% and reported no attack of pest and did not opt for any spray operation but the optimum productivity is gained for those who opted for one spray for the wheat crop with no pest attack reported. The farmers who did two spray operations for no pest attack also lost about 3.55% in productivity. The attack of pests on wheat crops emphasized that productivity loss is 21.56% (8.35 mds/acre) for no spray, and this gap is reduced to 2.24% and 1.78% for one and two spray operations. The analysis depicts that for the optimum productivity levels, the area share is nominal and needs to be enhanced to get the wheat output maximization.

3.8. Categorization of Factors Levels and Significance of Mean Difference

This study layout the good design for the most optimum plan for categorizing the agronomic constraints in view of wheat yield enhancement practices for true policies decision to attain food sustainability. Table 9 shows the categorization of factors levels and their significance of mean difference. Category-1 (Major loss factors), refer to those factors whose probability share (%) at optimum level of inputs lies in (1-25%) and these are urea for (125-175) kg/acre, DAP for (100-125) kg/acre, other fertilizers for 25 kg/acre, spray (2-3) for pest attack, one spray for no pest attack. The rest of the farmers are losing their productivity. Category-I are responsible for major loss as larger share of the formers are not using the best level and losing the productivity. Category-II (Medium loss factors), refer to those factors whose probability share (%) at optimum level of inputs lies in (26-50%) and these are reported four irrigations, harvesting for April (1-20) and certified seed (26.06%). The rest of the farmers are losing their productivity. Category-II is responsible for medium loss as medium share of the formers are not using the best level

Table 8. Interaction study for pest attack and spray operation.

Factors levels		Pest attack							
		Yes				No			
		R.F. (%)	Yield mds/acre	Abs. gap	Rel gap (%)	R.F. (%)	Yield mds/acre	Abs. gap	Rel gap (%)
No. of pest spray	0	4.32	30.37	8.35	21.56	75.2	35.72	1.42	3.82
	1	16.77	37.85	0.87	2.24	2.42	37.14	0	0
	2	1.16	38.03	0.69	1.78	0.10	35.82	1.32	3.55
	3	0.04	38.72	0.00	0.00				

Table 9. Categorization of factor levels and significance of means differences.

Factors levels	Probability share of farmers using the optimum/best level of inputs	Rest of the farmers who using non-optimum level of inputs	Category	Testing the significance of mean difference using ANOVA (F- Statistic)
Planting period November	83.03%	16.97	III	247.8**
Harvesting period April 1-20	44.47%	55.53	II	281.1**
Urea 125-75 kg	7.3%	92.7	I	634.4**
DAP (100-125) kg	4.44%	95.56	I	606.2**
Other fertilizers 25 kg	(0.64%)	99.36	I	33.5**
Irrigation/ 4 water	27.82%	72.18	II	795.8**
Certified seed	26.06%	73.94	II	149.3**
Soil type Chikny	66.30%	33.7	III	420.1**
Spray 2-3 with pest attack	1.2%	98.8	I	--
Spray 1 with No. pest attack	2.42%	97.58	I	--

** show the results are highly significant.

and losing the productivity. Category-III (Minor loss factors), whose probability share at optimum level of inputs lies in 51% and above is reported as planted in November and soil type. The rest of the farmers are losing their productivity. Category-III is responsible for minor loss as slight share of the formers are not using the best level and losing the productivity. Any rise in the probability ratio of area share for categories I to III at their optimum levels results in enhancement in wheat productivity, but in diminishing order. The value of F-statistic depicts that there is significance difference between the means of the groups for all the factors groups.

4. DISCUSSIONS

Food demand is rising worldwide, while its production is not enough to meet this demand. In Pakistan and neighboring countries, the majority of farmers are illiterate and they are applying different agronomic constrains in field experiments. They are unaware about the best level of factors. This research is supporting by the findings of Van-Ittersum and Cassman [17] that the yield gap analysis is one of the powerful measures to estimate production. Hatfield and Beres [18], Van-Dijk *et al*. [19] and Van-Oort *et al.* [25] also supporting this research methodologies by indicating that the yield gap analysis provides a practical and operational framework to assess the trend and average yield difference of crop. Similar studies were conducted by Islam [5], Islam [20], Qayyum [22] and also by Qayyum and Pervaiz [23], but these studies did not layout the categorization of agronomic constrain in scientific way. However, this research introduces new categorizations of agronomic factors to identify the optimal conditions for enhancing wheat productivity. A similar approach was employed by Hameed *et al*. [26] for the cotton crop, whereas our study focuses on wheat crop. The concept for categorizations of agronomic factors identified in the current study is practically applicable for sustainable precision agriculture and policy decisions.

5. CONCLUSIONS

The demand for wheat as a primary food crop is increasing at an accelerated rate to meet the needs of the expanding population. In south Asian countries like Pakistan, majority of farmers are illiterate and they are applying different non-optimal agronomic constrains in field. The research introduced the new concept of categorizations of agronomic factors into high, medium and low risk (loss) factors, liable to identify the reason of low wheat yield and to make the policies for the enhancement practices of wheat productivity in Pakistan. The 26430 crop cut experiments are statistical analyzed to determine the optimum levels of individual levels and their interactions effects. Category-1 (Major loss factors) refer to those factors, whose share at better level of output falls in (1-25%) and these are urea (7.3%) for (125-175) kg/acre, DAP (4.44%) for (100-125) kg/acre, other fertilizers (0.64%) for 25 kg/acre, Spray (2-3) for pest attack (1.2%), one spray for no pest attack (2.42%). Category-I are major risk factors as larger share of the formers are not using the best level and losing the productivity. Category-II (Medium loss factors) refer to those factors, whose share at a better level of output lies between (25.1-50%) and these are four irrigations (27.82%), harvesting period (44.47%) for April (1-20) and certified seed (26.06%). Category-II is responsible for medium loss. Category-III (Minor loss factors) refer to those factors, whose share at a better output level is above 50.1% and these are planting period November 87.03% and soil type 66.30% for chikny type. Category-III is responsible for minor loss as maximum farmers is already using the best level of inputs. There is a foremost need to enhance the relative share of factors for category-1, which can rapidly enhance the productivity, while the increased relative share of category-II will also enhance the productivity, but it will be below from category-1. A rise in the relative share of factors for category III increases the productivity, but less from categories I and II. There is a foremost need to apply the factors, levels and their interaction at their optimum output level to meet the yield gap and ensure the food security and its availability concerns in the region. This study may layout the design to formulate direction ordinated policies decision in view of wheat productivity enhancement practices to attain food sustainability through precision agriculture.

6. CONFLICT OF INTEREST

The author declares no conflict of interest.

7. ACKNOWLEDGEMENTS

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Supplementary Data

Risk Factors Evaluation and Antiviral Eradication Therapies Among HCV Infected Family Members of Northern Regions, Pakistan

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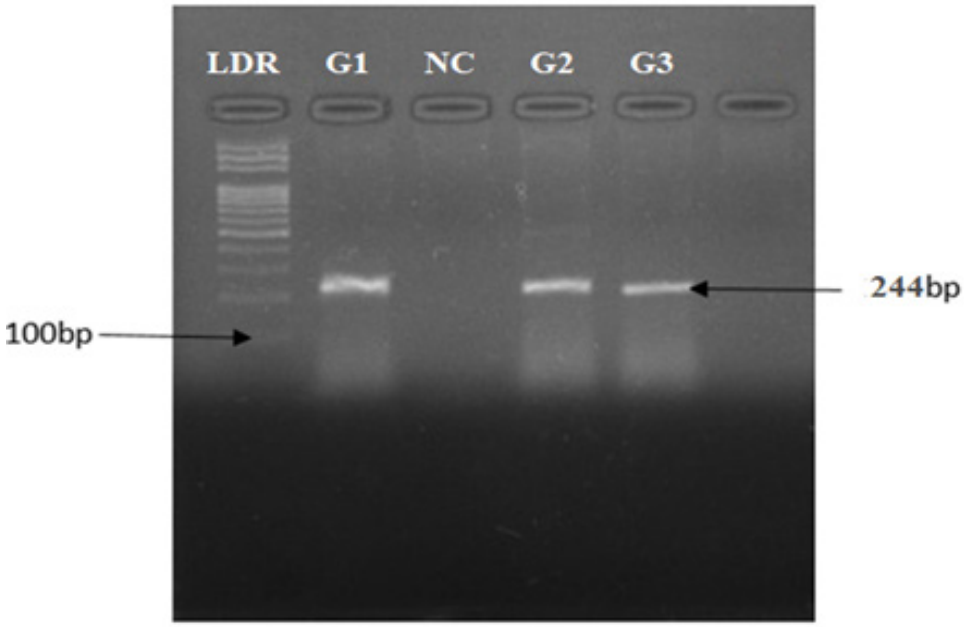

Supplementary Fig. S1. Target DNA bands on agarose gel.

* Corresponding Author: Hamza Altaf <hamza.altaf764@gmail.com>

Suplementary Table 1. Association of demographic, socioeconomic, and education level with HCV infection among HCV-positive families.

Variables	Infected family members	HCV infection status n(%)	Odd ratio (95%CI)	*P* value
Number of rooms				0.061
1-4	115	49 (42.6)	1.96 (0.90-1.01)	0.105
5-8	08	06 (75.0)	0.75 (0.50-1.11)	0.429
9-12	01	04 (100.0)	1.00 (1.00-1.00)	1.000
Number of people				0.334
1-5	48	25 (52.1)	1.67 (0.78-3.54)	0.179
6-10	66	26 (39.4)	1.00 (1.00-1.00	1.000
11-16	11	06 (54.5)	0.54 (0.15-1.95	0.345
Education level				0.007
Illiterate	69	39 (56.5)	1. 27 (1.31-5.72)	0.007
Literate	56	18 (32.2)	1.00 (1.00-1.00)	1.000
Monthly income				0.073
5000-20000	86	47 (54.7)	3.61 (0.36-36.15)	0.245
21000-50000	35	09 (25.7)	1.03 (0.09-11.29)	0.975
51000-90000	04	01 (25.0)	1.00 (1.00-1.00)	1.000

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